Delineating the biology of Anaplastic Lymphoma Kinase (ALK) and its resistance to crizotinib

by

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Abstract

Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase receptor that was initially identified as a potent oncogenic driver in anaplastic large-cell lymphoma (ALCL) in the form of NPM-ALK fusion protein. Various forms of oncogenic ALK proteins have been identified, subsequently, in various types of human cancers. The focus of this thesis was to get more insight into the ALK biology and how this could impact cancer phenotypes.

1) Crizotinib is an ALK inhibitor that has been found to be therapeutically useful against a subset of ALK-positive tumors. However, clinical resistance to this drug has been well known and the mechanism of this resistance is incompletely understood. In this part of my thesis, I hypothesized that the crizotinib—ALK binding is a determining factor for the sensitivity to crizotinib in ALK-positive cell lines derived from various types of cancers. Using the cellular thermal shift assay (CETSA), I measured the crizotinib—ALK binding in a panel of ALK-positive cell lines, and correlated the findings with the ALK structure and its interactions with specific binding proteins. I found that the biological response to crizotinib treatment significantly correlated with crizotinib—ALK binding. Additionally, I found that the crizotinib-resistant cell lines expressed higher protein levels of β-catenin. Furthermore, the siRNA knockdown of β-catenin in these cells restored crizotinib—ALK binding and was associated with a significant lowering of IC_{50}. Taken together, this part of the thesis showed that the crizotinib—ALK binding measurable by CETSA is useful in predicting crizotinib sensitivity in ALK-positive cancer cells and crizotinib—ALK binding is dictated by the structure of ALK and some of its binding partners.

2) Since I have highlighted the importance of the physical binding between crizotinib and ALK in determining the crizotinib sensitivity, here I asked if these observations hold true for the stem-like cells in neuroblastoma (NB) cells, which were purified based on their responsiveness to a Sox2 reporter. NB is the most common pediatric extra-cranial solid tumor and the survival for metastatic NB remains <50% despite intensive multi-modality therapies. Notably, the sensitivity of NB cells to crizotinib is highly variable. In this study, I found that compared to bulk, reporter unresponsive (RU) cells, reporter responsive (RR) cells had significantly higher neurosphere formation ability, expression of CD133/nestin, and chemo-resistance. Using CETSA, I found that RR cells exhibited higher crizotinib resistance than RU cells and show no crizotinib—ALK binding. The absence of crizotinib—ALK binding in RR cells can be attributed to their high β-catenin expression, since siRNA knockdown of β-catenin restored the crizotinib—ALK binding and lowered the crizotinib IC_{50} to the level of RU cells. Importantly, enforced expression of β-catenin in RU cells resulted in the opposite effects. Therefore, high expression of β-catenin in the stem-like NB cells contributes to their crizotinib resistance.

3) The mechanisms by which the expression of full-length ALK (wild-type or mutant), which is detectable in the majority of NB cases, are regulated are not well understood. I have identified a novel *ALK* transcript characterized by the retention of the entire sequence of intron 19 (i.e. *ALK-I19*). *ALK-I19* was detected in the four NB cell lines, used in the previous section, and in one-third of patient samples. In this study, I provided a description of this ALK variant. Specifically, the functional significance of *ALK-I19* was determined by specific siRNA knockdown of this transcript, which resulted in substantially decreased expression of the fully-spliced *ALK* transcripts (i.e. *FS-ALK*) and ALK protein, as well as a significant reduction in cell growth. Clinically,

ALK-I19 expression correlated with undifferentiated histology and strong ALK protein expression, detectable by immunohistochemistry in >50% of the tumors. Importantly, patients with tumors that did not express *ALK-I19* and lacked *MYCN* amplification had an excellent clinical outcome, with 19/19 patients survived at 5-years as compared to only 10/18 patients with tumors carrying *ALK-I19* and/or *MYCN* amplification. This data suggests that the absence of *ALK-I19* and *MYCN* amplification is a useful prognostic marker for NB patients.

To conclude, this thesis has provided insights into the molecular mechanism underlying the biology of ALK and a novel mechanism of resistance, which may provide valuable therapeutic targets in neuroblastoma and other ALK-positive cancers.

Preface

This thesis represents collaborative work, led by Professor Raymond Lai at the University of Alberta.

Chapter 2 of this thesis has been published as:

Alshareef A, Zhang HF, Huang YH, Wu C, Zhang JD., Wang P, El-Sehemy A, Fares M, Lai R. The use of cellular thermal shift assay (CETSA) to study Crizotinib resistance in ALK-expressing human cancers. *Scientific Reports* **2016,** *6*, 33710. As first author of this paper I prepared the first draft and revisions based on the suggestions and comments of the co-authors. I designed and performed most of the experiments described herein, except for the following: Zhang HF, Huang YH and Wu C contributed to the data shown in Figure 2.6, Figure 2.7C and Figure 2.8, and final review of the manuscript. Zhang JD. and Wang P generated the two crizotinib-resistant ALK+ALCL cell lines. El-Sehemy A and Fares M prepared Figure 2.13 and wrote the computational analysis. R.L. provided numerous comments and final review of the manuscript before it was submitted for publication.

Chapter 3 of this thesis has been submitted for publication as:

Alshareef A, Gupta N, Zhang HF, Wu C, Lai R. High expression of β-catenin is responsible for the crizotinib resistance in the small subset of stem-like neuroblastoma cells. (2017, Submitted). As first author of this paper I prepared the first draft and revisions based on the suggestions and comments of the co-authors. I designed and performed most of the experiments described herein, except for the following: Gupta N, Zhang HF and Wu C contributed to the data shown in Figure 3.1 and Figure 3.2, and final review of the manuscript. R.L. provided numerous comments and final review of the manuscript before it was submitted for publication.

Chapter 4 of this thesis has been submitted for publication as:

Alshareef A, Irwin M, Gupta N, Zhang HF, Haque M, Findlay S, Rayis M, Al-Dandan S, Lai R. The absence of a novel intron 19-retaining ALK transcript (*ALK-I19*) and *MYCN* amplification correlates with an excellent clinical outcome in neuroblastoma patients. (2017, Submitted). I was first author of this paper. I prepared the first draft and revisions based on the suggestions and comments of the co-authors. I designed and performed most of the experiments described herein, except for the following: Irwin M and Gupta N helped with the statistical analysis shown in Figure 4-12 and Figure 4-13, Zhang HF, Haque M and Findlay S helped with the development of the protocol for *ALK-I19* primer design and detection, Rayis M and Al-Dandan S provided sectional tissue slides, clinical data and diagnosis. R.L. provided numerous comments and final review of the manuscript before it was submitted for publication.

Archival patient tissue samples prepared as sectional tissue on slides from the King Fahad Medical City (KFMC), Saudi Arabia, used for this study, was approved by the Institutional Review Board of the KFMC. Written informed consent was obtained from all surgical patients to use resected samples for diagnostic testing and research. Moreover, the grant that supported this research project, of which this thesis is a part, also received research ethics approval from the University of Alberta Research Ethics Board, Project Name: “Study the oncogenic properties of ALK in human ALK^+ cancers"; application number: Pro00062737; Date: May 31, 2016.

Dedication

In the name of Allah,
the Most Gracious, the Most Merciful

To my parents
Mohammed Altaib Alshareef and
Mariam Saif

To my wife Noha Alansari
and my two lovely kids Bader and Bassam

To my brother Badr Alhadraniy

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List of Abbreviations

ABL - Abelson murine leukemia viral oncogene homology 1

ALCL - Anaplastic large cell lymphoma

ALK - Anaplastic lymphoma kinase

ALK-I19 - Anaplastic lymphoma kinase - Intron 19

ALK^{+}ALCL - anaplastic lymphoma kinase positive anaplastic large cell lymphoma

ALL - acute lymphoblastic leukemia

ALO17 - ALK lymphoma oligomerization partner on chromosome 17

APC - adenomatous polyposis coli

ATIC - 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase

ATP - Adenosine triphosphate

BAD - Bcl2-associated death receptor

BC - breast cancer

BCA - bicinchoninic acid

BCL2 - B-cell lymphoma 2

BCR - breakpoint cluster region

BCR-ABL - breakpoint cluster region fused to Abelson kinase

C - cytosine

C2orf44 - chromosome 2 open-reading-frame 44

CARS - cysteinyl-tRNA synthetase

CDC25A - cell division cycle 25 homolog A

CETSA - cellular thermal shift assay

CHOP - cyclophosphamide, hydroxydaunorubicin, oncovin, prednisone

CLTC - clathrin heavy chain

CML - chronic myelogenous leukemia

c-MYC - V-myc avian myelocytomatosis viral oncogene homolog

CNS - Central nervous system

Co-IP - co-immunoprecipitation

CRC - colorectal carcinoma

CSCs - cancer stem cells

C-terminal - carboxy terminal

DLBCL - diffuse large B-cell lymphoma

ECL - enhanced chemiluminescence

EGFR - epidermal growth factor receptor

ELISA - enzyme-linked immunosorbent assay

EML4 - echinoderm microtubule-associated protein-like 4

EML4-ALK - echinoderm microtubule-associated protein-like 4 fused to anaplastic lymphoma kinase

ER - estrogen receptor

ERK - extracellular signal-related kinase

ESCC - esophageal squamous cell carcinoma

EV - empty vector

F - phenylalanine

FACS - fluorescent activated cell sorter

FAM150 - Family with sequence similarity 150

FBS - fetal bovine serum

FDA - Food and Drug Administration

FGF - Fibroblast growth factor

FISH - fluorescence *in situ* hybridization

FS-ALK – fully spliced Anaplastic lymphoma kinase

G - guanine

Grb2 - growth factor receptor-bound protein 2

GSK - glycogen synthase kinase

GSK-3α/β - glycogen synthase kinase 3-alpha/beta

H&E - Hematoxylin and eosin

HEK - human embryonic kidney

HGFR - hepatocyte growth factor receptor

HSP - Heat shock protein

IBC - inflammatory breast cancer

IC - inhibitory concentration

IGF-1R - insulin-like growth factor 1 receptor

IHC - immunohistochemistry

IL - interleukin

IMT - inflammatory myofibroblastic tumor

IP - immunoprecipitation

IP3 - inositol-1,4,5-triphosphate

IR - Insulin receptor

IRS-1 - insulin receptor substrate 1

JAK - Janus kinase

Jeb - Jelly Belly

JNK - Jun-amino terminal kinase

KIF5B - kinesin family member 5B

LC/MS - liquid chromatography-mass spectrometry

Lck - lymphocyte protein tyrosine kinase

LDLa - Low density lipoprotein class A

LEF - lymphoid enhancer factor

LTK - leukocyte tyrosine kinase

mAB - monoclonal antibody

MAM - Meprin A-5 protein and receptor protein tyrosine phosphatase Mu

MAPK - mitogen-activated protein kinase

MAX - MYC-associated factor X

MCL - mantle cell lymphoma

Mcl1 - myeloid cell leukemia sequence 1

MEK - mitogen-activated protein kinase

MET - Mesenchymal-epithelial transition

MK - midkine

MMR - mismatch repair

MSH - MutS homolog

MSN - Moesin

mTOR - mammalian target of rapamycin

MTS - 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4sulfophenyl)-2H-tetrazolium

MYH9 - myosin heavy chain 9

NB - neuroblastoma

NHL - non-Hodgkin lymphoma

NIPA - nuclear interaction partner of ALK

N-MYC - neuroblastoma MYC Oncogene

NPM - nucleophosmin

NPM-ALK - nucleophosmin fused to anaplastic lymphoma kinase

NRAS - neuroblastoma RAS viral (v-ras) oncogene homolog

ns - not significant

NSCLC - non-small cell lung cancer

N-terminal - amino terminal

OS - overall survival

P/p - phospho/phosphorylation p-Y – phosphorylated tyrosine

PAGE - Polyacrylamide gel electrophoresis

PARP1 - poly (ADP ribose) polymerase 1

PBS - phosphate buffered saline

PC12 - Pheochromocytoma 12

PCNA - proliferating cell nuclear antigen

PCR - polymerase chain reaction

PDB - Protein data bank

PDGFR - platelet derived growth factor receptor

PFS - progression-free survival

PHOX2B - paired-like homeobox 2b

PI - propidium iodide

PI3K - phosphatidylinositide 3-kinase

PIP2 - phosphatidylinositol 4,5-bisphosphate

PKC - protein kinase C

PLC-γ - phospholipase C gamma

PPFIBP1 - protein tyrosine phosphatase receptor type F polypeptideinteracting protein-binding protein 1

PTB - phosphotyrosine binding

PTEN - phosphatase and tensin homolog

PTN - pleiotrophin

PTP - phosphotyrosine phosphatase

PTPN11 - protein tyrosine phosphatase, non-receptor type 11

RANBP2 - Ras-related nuclear protein- binding protein 2

RAS - Rat sarcoma

RCC - renal cell carcinoma

RET - Rearranged during transfection

ROS1 - c-ros oncogene 1

RR - reporter responsive

RTK - receptor tyrosine kinase

RT-PCR - reverse transcriptase PCR

RU - reporter unresponsive

SCID - severe combined immunodeficiency

SDS - Sodium dodecyl sulphate

SDS-PAGE - sodium docedyl sulfate-polyacrylamide gel electrophoresis

SH2 - Src homology 2

SHC - Src homology region 2 domain containing

SHH - sonic hedgehog

SHP1 - Src homology region 2 domain-containing phosphatase-1

siRNA - small interfering RNA

Sox2 - SRY (sex determining region Y)-box 2

SQSTM1 - sequestosome 1

SRC - v-Src avian sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog

SRR2 - Sox2 regulatory region 2

STAT - signal transducer and activator of transcription

T - total, or thymine

t - translocation

TAGC - The Applied Genomics Centre

TCF - T-cell factor

TFG - *TRK*-fused gene

TKD - tyrosine kinase domain

TKI - Tyrosine kinase inhibitor

TNBC - triple-negative breast cancer

TPM3/4 - Tropomyosin 3/4

Trk A/B - Tropomyosin receptor kinase A/B

v - variant

Y - tyrosine

ZNF2 - zinc finger protein 2

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Chapter 1

General Introduction

1.1 The Tyrosine Kinase superfamily

Phosphorylation of proteins is the most common class of post-translational modification used in signal transduction (1, 2). Protein phosphorylation occurs at serine, threonine, tyrosine, histidine, arginine, or lysine residues (1). The human protein tyrosine kinase family consists of 90 genes (which represents 17% of the protein kinase family), 58 genes encode for receptors and 32 genes encode for non-receptor tyrosine kinases (3). Protein tyrosine kinases play a major regulatory role in almost every aspect of cell biology including proliferation, differentiation, apoptosis, immune response, nervous system function, and transcription (1). A variety of diseases, such as cancer, diabetes, autoimmunity, cardiovascular, inflammatory, and nervous system disorders, occur due to dysregulation of protein kinases (3). Extensive effort has been expended to determine the physiological and pathological functions of protein tyrosine kinase signal transduction pathways during the past 40 years (4, 5).

Protein tyrosine kinases represent ideal targets for therapy and the development of tyrosine kinase inhibitors (TKIs) has advanced the field of cancer treatment in making cancer a manageable disease (4, 6). The first cancer therapy to show the potential for such targeted action was imatinib mesylate (Gleevec), which targeted the tyrosine kinase BCR-ABL in chronic myeloid leukemia (CML) (6, 7). Subsequent research established further promising tyrosine kinase targets, such as EGFR (epidermal growth factor receptor) and ALK (anaplastic lymphoma kinase), and their corresponding inhibitors are currently being used in the treatment of cancer patients (8, 9).

1.1.1 Regulation of the activity of receptor tyrosine kinases

The typical feature of protein tyrosine kinases is that they are stringently regulated, and the mechanisms for the interconversion of dormant and active enzymes are often intricate (10). Ligands binding to the extracellular domain usually activates receptor tyrosine kinases (RTKs) by inducing receptor dimerization or oligomerization (11). When an RTK is dimerized or oligomerized, the main phosphorylated product is the receptor itself by the process of autophosphorylation (11). Upon RTK autophosphorylation, numerous cytoplasmic proteins that have SH2 (Src homology region 2) or PTB (phosphotyrosine-binding) domains recognize tyrosine phosphorylated residues and have intrinsic enzymatic activity, such as Src, or adapter proteins that

recruit other enzymes, such as Grb2 (12). Next, signal propagation is amplified and regulate the majority of cellular processes such as cell cycle regulation, cell shape, movement, proliferation, differentiation, and survival (12).

1.2 Anaplastic Lymphoma Kinase (ALK)

Anaplastic Lymphoma Kinase (ALK) is a receptor tyrosine kinase belonging to the insulin receptor (IR) superfamily (NCBI Gene ID: 238, Uniprot query Q9UM73) (13). Importantly, key residues essential for catalytic activity are conserved among receptor tyrosine kinases (13). ALK is highly conserved among species since human and mouse ALK proteins share 85% identity (14). Among kinases, ALK shares the greatest homology with leukocyte tyrosine kinase (LTK) (i.e 57% amino acid identity and 71% similarity in their overlapping region in the whole protein) (14).

1.2.1 ALK structure

The well-studied and characterized *ALK* gene encodes for a 177 kDa protein that undergoes N-linked glycosylation, resulting in the 220 kDa mature protein that is expressed on the cell surface and capable of being tyrosine phosphorylated (15). Structurally, ALK comprises of an extracellular domain, a transmembrane domain, and an intracellular domain that includes the tyrosine kinase region (16). The extracellular domain of ALK consists of a signal peptide, two MAM (Meprin/A5-protein/PTPmu) domains, one LDLa (Low-density lipoprotein receptor domain class A) domain, and a glycine-rich domain (17) (**Figure 1.1**). Each MAM domain is comprised of ~160 amino acids that are present in transmembrane proteins, such as receptor protein-tyrosine phosphatases, where they appear to have adhesive functions (18, 19).

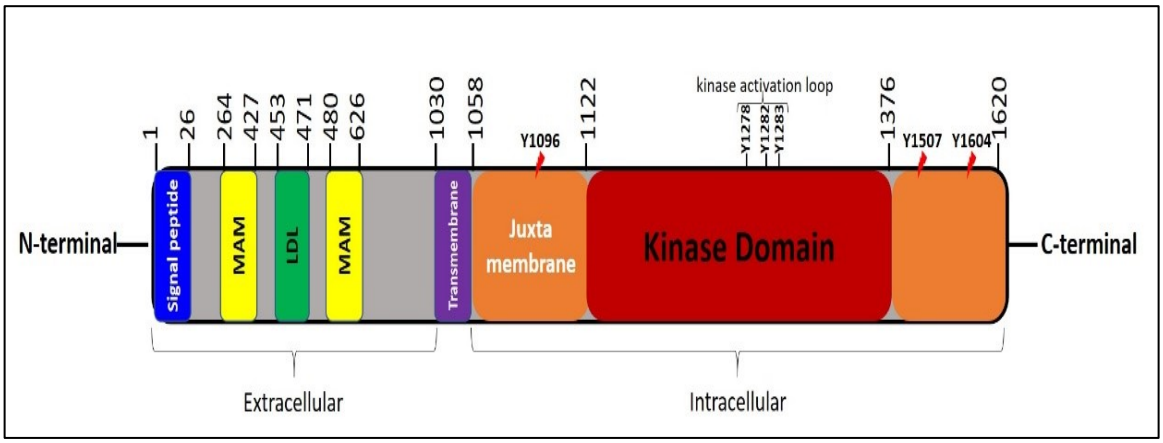

Figure 1.1 Domain structure of ALK.

The N-terminal, extracellular region of human ALK contains the following domains: a signal peptide (aa 1–26, blue), two MAM domains (aa 264–427 and 480–626, yellow), one LDL domain (aa 453–471, green) and a glycine-rich domain (aa 816–940, gray). The transmembrane domain (TM) (aa 1030–1058, purple) connects the extracellular and intracellular domains. The intracellular (cytoplasmic) domain contains the juxtamembrane (1058–1122, orange) and the tyrosine kinase domain (aa 1122–1376, red). Y1096, Y1507 and Y1604 are ALK residues that serve as docking sites that bind SH2/PTB proteins which participate in ALK-mediated signal transduction. MAM: meprin/A5-protein/PTPmu; LDL: low-density lipoprotein receptor domain class A.

1.2.2 ALK ligands

In *Drosophila melanogaster*, *dAlk* was shown to be activated by its ligand Jelly belly, thereby regulating neuronal circuitry within the visual system and the development of the gut musculature (20, 21). In mammals, initial studies have reported two ligands that activate ALK, pleiotrophin (PTN) and midkine (MK) (22, 23). However, several reports show contradictory results that PTN and MK do not mediate ALK activation (24-26). More recently, *Regairaz et al* showed that MK can lead to ALK phosphorylation, however, whether the MK-ALK binding is direct or through a mediator is not completely understood (27). Therefore, MK and PTN have not been accepted as true ligands of ALK since contradictory reports have been published. Two other ligands, Augmentor α and β, were recently reported as specific ligands for ALK and LTK (28). While Augmentor β (also known as FAM150A) was shown to bind weakly to ALK, Augmentor α was shown to bind to and robustly activate ALK (29).

1.2.3 ALK expression and function in normal cells

1.2.3.1 Normal expression

Initial studies of human *ALK* mRNA expression demonstrated the presence of 4 *ALK* transcripts (4.0, 6.0, 6.5 and 8.0 kilobase or kb) in normal tissues, mainly in the central and peripheral nervous systems, with no or very minimal expression in other tissues (30). Subsequent work has further characterized the 6.5 kb ALK, which is a 6226 base pair (bp) transcript, while the other transcripts have not been characterized yet (31). RNA *in situ* hybridization studies showed that ALK expression is predominantly expressed in specific regions of the developing mouse brain and peripheral nervous system, such as the thalamus and ganglia, beginning at day 11 of embryogenesis (32). After birth, the levels of ALK mRNA decreased substantially and were detected at only very low quantities in neonates as shown by immunoblotting studies (32-34). This finding was also confirmed in adult human central nervous system (CNS) tissue samples where low levels of ALK were detected by immunohistochemical studies (35). In *Drosophila melanogaster*, *dAlk* was shown to be expressed in the developing embryonic mesoderm and CNS (36).

It has been reported that the full-length ALK receptor is expressed as two forms corresponding to 140 and 220 kDa (25, 37, 38). The 140 kDa form has been suggested to be a cleaved form of ALK that resulted from an extracellular cleavage of the 220 kDa form by a yet-to-be identified mechanism (25). There is some experimental evidence that Schwann cells release a factor or factors, yet-to-be identified, that control the inhibition of this cleavage (33). While the nature of the 140 kDa ALK form has not yet been comprehensively studied, one study shows that this form is localized at the cell surface (39), but the biological importance of the cleavage is still unknown.

1.2.3.2 Normal function

Although the normal function of the full-length ALK receptor is not entirely clear, the observation that ALK expression is mainly restricted in the nervous system suggests that ALK plays an important role in the development of the brain and exerts its effects on specific neurons in the nervous system (40). Studies performed in mice showed no obvious anatomical abnormalities and a normal life-span upon homozygous deletion of the ALK kinase domain (41). Nevertheless, these mice do exhibit an 'antidepressant profile' including an age-dependent increase in basal hippocampal progenitor proliferation, increased basal dopaminergic signaling within the prefrontal cortex and enhanced performance in novel object recognition/location tests (41). Double knockout of ALK and its closest family member, leukocytes tyrosine kinase (LTK), in mice has also been reported to be viable (41-43). In chick, ALK was identified to be expressed and control the sympathetic neuron proliferation (44). Specifically, this study showed that the ectopic overexpression of either wild type or mutated *ALK* resulted in proliferation of the premature neurons, while downregulation of *ALK* caused decrease in sympathetic neuron proliferation (44). A more recent study showed that the co-expression of *MYCN* and activated ALK^{F1174L} is required for maintaining the proliferation of premature neurons in chick (45). In zebrafish model, one study showed that *alk* is strongly expressed in the developing nervous system, especially in the brain (46). Additionally, overexpression of *alk* caused proliferation and deregulated neurogenesis, while downregulation of *alk* caused apoptosis of cells and prevented neuronal differentiation (46).

1.3 ALK in cancer

ALK got its name because it was first described, in 1994, in a subset of anaplastic large cell lymphoma (ALCL), a subtype of peripheral T-cell lymphoma (30, 47). A subset of ALCL harbors the (2;5) chromosomal translocation, such that the amino terminus of nucleophosmin (NPM) is linked to the catalytic domain of the ALK protein, resulting in constitutive activation of the ALK kinase (30). In 2007, echinoderm microtubule-associated protein like 4 (EML4)-ALK oncoprotein was identified in non-small cell lung cancers (NSCLCs) (48, 49). The *ALK* locus has recently been identified as a hotspot for activating translocation events in many types of cancer, with over 22 different translocation partners identified (16, 50).

In 2008, *ALK* was shown to be a critical oncogene in the pathogenesis of neuroblastoma (NB), the most common and aggressive childhood solid tumor (51-54). *ALK* amplification accounts for 3.5% of NB patients while activating mutations account for 8.4% (54). A study showed that elevated *ALK* mRNA levels are associated with an unfavorable neuroblastoma phenotype independent of the genomic *ALK* status (55). In other words, patients with *ALK*-wild-type^{high} tumors in which *ALK* expression was as high as in *ALK*^{mutated} tumors had a similar poor outcome as those with *ALK* mutations. Importantly, the presence of an ALK mutation or overexpression displays a similar genetic profile by microarray (55). This suggests high ALK activity, independent of mutation, can contribute to NB development (56). Inhibition of ALK leads to the cell cycle arrest and apoptosis, supporting the concept that ALK is necessary for the development and progression of cancer (57).

1.3.1 Oncogenic ALK signaling

Multiple signaling pathways have been shown to be activated through NPM-ALK, the most comprehensively studied form of ALK, including Signal Transducer and Activator of Transcription (STAT), Wnt, and other signaling cascades, which affect cell growth, transformation and antiapoptotic signaling (17, 57-60) (**Figure 1.2**). Multiple signaling pathways were shown to be activated through ALK, including phospholipase Cγ (PLCγ), Janus kinase (JAK)-STAT, PI3K-AKT, mTOR, Wnt, sonic hedgehog (SHH), interleukins (ILs), JUNB, Myc, CRKL-C3G (also known as RAPGEF1)-RAP1 GTPase and MAPK signaling cascades, which affect cell growth, transformation and antiapoptotic signaling (17, 57-59, 61). Additionally, many adaptors were

shown to be involved in ALK signaling such as Suc1-associated neurotrophic factor target 2 (SNT2; also known as FRS3)-fibroblast growth factor receptor substrate 2 (FRS2), insulin receptor substrate 2 (IRS2), SHC and growth factor receptor-bound protein 2 (GRB2). ALK also affects more downstream targets, such as BCL-2-interacting mediator of cell death (BIM; also known as BCL2L11) and cyclin D2, which are essential components for promoting cell survival and growth (17, 57-59, 61).

There are many other interesting - but lesser characterized - targets of ALK including nuclear interacting partner of ALK (NIPA; also known as ZC3HC1) (62, 63), the small GTPases RAC1, cell division control protein 42 (CDC42) (64, 65), p130CAS (also known as BCAR1) (66), SHP2 (also known as PTPN11), Src (67-69), and the lipid kinase FYVE finger-containing phosphoinositide kinase (PIKFYVE) (70). Novel ALK targets have been identified by proteomics efforts, leading to a substantial list of potential players in ALK signaling (68, 71-79). This list is complemented by transcriptome-based approaches that have identified several ALK-regulated genes (80). For example, looking at the activated full-length ALK, *MYCN* has been identified as a transcriptional target from recent work in the neuroblastoma field (81). Further oncogenic ALK signaling will be covered in the next section.

1.3.2 ALK-interacting proteins

As summarized in the previous section, ALK is known to promote tumorigenicity by binding to and constitutively activating many cellular signaling proteins (60). Although a trove of valuable information has been published on NPM-ALK signaling, much less is known about ALK-interacting proteins by the other ALK fusions or the mutated/amplified full-length ALK receptor (40). ALK has been shown to interact with a wide range of oncogenic molecules, several of which are discussed in this section.

1.3.2.1 STAT3

STAT3 is one of a family of 7 STATs that comprise latent transcription factors (82). Phosphorylation and subsequent activation of STAT3 occurs either via a family of 4 cytokine receptor-associated tyrosine kinases known as JAKs, via its interaction with RTKs such as ALK

and EGFR or via its interaction with cytoplasmic tyrosine kinases such as Src and Abl (60). STAT3 is one of the proteins that was most significantly tyrosine-phosphorylated in response to ALK activation in both the full-length ALK and ALK fusion proteins (83). Specifically, it has been shown that full-length ALK as well as NPM-ALK fusion promote the tyrosine phosphorylation of STAT3 on one of its tyrosine residues, Y705, which is critical for the dimerization and activation of STAT3 (83, 84).

In the ALK^{+}ALCL cancer model, NPM-ALK binds to, phosphorylates and activates STAT3, which has been shown to be central to the NPM-ALK─mediated tumorigenesis (60, 84-86). For instance, immortalized mouse embryonic fibroblasts with normal expression of STAT3 were transformed by NPM-ALK, whereas *STAT3* gene knockout dramatically decreased the malignant transformation by NPM-ALK (85). One study reported an interaction of STAT3 with full-length ALK when overexpressed; however, this interaction was not detectable at the level of endogenous proteins in neuroblastoma cell lines (cell lines include CBL-GA, which carries ALK^{R1275Q}; CLB-GE, which carries ALK^{F1174V}; Kelly, which carries ALK^{F1174L} and CLB-BAR, which carries $ALK^{\Delta exon4–12}$) suggesting that care should be taken when interpreting these results (83). In other words, whether ALK can interact with STAT3 is most likely cell line dependent. Therefore, whether STAT3 is involved directly or indirectly in ALK-mediated tumorigenesis in neuroblastoma as well as other type of cancers that carry other ALK fusions/forms still needs further investigation.

1.3.2.2 β-catenin

β-catenin protein can be found in two separate pools in the cell and is referred to as a bifunctional protein (87). In the first pool, β-catenin plays as an adhesion molecule bound to E-cadherin and α-catenin within adheren junctions at the plasma membrane (87). In the second pool (also known as the cytosolic soluble pool), it plays as a key intermediate in the canonical Wnt signaling pathway and functions as a transcription factor (87). It is still questionable whether the two functions of β-catenin, cell adhesion and transcriptional activation, are related (88).

The majority of β-catenin studies focus on its role as a critical transcription factor. In the absence of Wnt molecules, the β-catenin remains in a cytoplasmic protein complex [comprised of

adenomatous polyposis coli (APC) and Axin], where it is phosphorylated initially by casein kinase I, and then by the serine/threonine kinase glycogen synthase kinase-3 (GSK-3). Phosphorylated β-catenin targets it for ubiquitination and proteasomal degradation (89). The binding of Wnt ligands to their cell-surface receptor complex consisting of Frizzled (a family of 10 G protein-coupled receptor proteins) and LRP5/6, activates the cytoplasmic phosphoprotein disheveled and inhibits GSK-3β-mediated phosphorylation of β-catenin. This inhibition of phosphorylation induces the stabilization and accumulation of cytoplasmic β-catenin and its translocation into the nucleus, where it activates the transcription factor T-cell factor (TCF)/lymphoid enhancer factor (LEF) and stimulates the transcription of target genes such as *c-Myc* and *CCND1* (89, 90).

The β-catenin-mediating Wnt signalling pathway plays crucial roles in embryonic development as well as tissue homoeostasis in adults (89). Thus deregulation of this pathway is linked to a variety of human diseases, including cancer (91). β-catenin was also shown to be an important signaling protein leading to drug resistance in many cancer types (92, 93), although, the mechanism behind its ability to mediate drug resistant is largely unknown. For example, β-catenin targeting causes abrogation of tyrosine kinase resistance in BCR-ABL, the oncogenic tyrosine kinase expressed in chronic myeloid leukemia (93).

In 2011, β-catenin was shown to be constitutively active in ALK^+ALCL cells and its downregulation, using siRNA, resulted in a significant reduction in cell growth (94). In this study, NPM-ALK was shown to interact with and to upregulate β-catenin transcriptional activity via a yet-to-be defined mechanism, however, it did not regulate the protein level, nuclear localization or tyrosine phosphorylation of β-catenin in ALK^+ALCL cells (94). These observations regarding the relationship between β-catenin and NPM-ALK are not similar with that between β-catenin and BCR-ABL. Particularly, BCR-ABL was shown to stabilize β-catenin by increasing its tyrosine phosphorylation, nuclear translocation, and transcriptional activity (95).

The relationship between β-catenin and other ALK forms is still lacking. However, there are a few reports highlighting the biological significance of targeting β-catenin in cancer types that may carry ALK aberrations such as neuroblastoma, NSCLCs and medulloblastomas (96-98). In one study, β-catenin knockdown effectively reduced the growth of neuroblastoma cells *in vitro* and in nude mice (99). Two other reports observed enhanced cell viability of two NB cell lines upon

overexpression of mutated β-catenin compared to cells expressing the empty vector (100, 101). For instance, β-catenin overexpression has been shown to be associated with resistance to gefitinib, an EGFR inhibitor, in NSCLC (102). During the past few years, many studies have focused on discovering small molecule inhibitors capable of disrupting β-catenin protein-protein interaction, thereby downregulating β-catenin function in cancer cells (103-105). For example, PRI-724, which inhibits the recruiting of β-catenin with its coactivator CBP (the binding protein of the cAMP response element-binding protein CREB), showed promising results from a phase I trial (106).

1.3.2.3 PI3K/Akt

Activation of class-Ia phosphoinositide 3-kinase (PI3K) occurs as a result of receptor and non-receptor tyrosine kinase activation (107). PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) and this leads to the creation of the lipid second messenger phosphatidylinositol-3,4,5-triphosphate (PIP3) (107). Subsequently, PIP3 contributes to the recruitment to the plasma membrane of a wide range of downstream targets, including Akt, a serine/threonine protein kinase also known as protein kinase B (PKB) (108).

A good number of studies showed that NPM-ALK interacts directly as well as indirectly with PI3K, implicating the PI3K/AKT pathway in NPM-ALK-mediated oncogenic transformation (59, 109, 110). PI3K pharmacologic inhibitors have been shown to induce apoptosis in NPM-ALK–expressing lymphoma cells (110). In one study, the use of PI3K/AKT/mTOR pathway inhibitors was shown to ameliorate the effect of crizotinib treatment in cell lines and in transgenic mice bearing ALK^{F1174L}/MYCN-positive neuroblastoma (111). Another study using neuroblastoma cell lines that harbor full-length ALK^{wt}, or ALK^{F1174V} or $ALK^{\Delta 4\text{-}11}$ showed that PI3K activity is required for ALK-mediated activation of extracellular signal–regulated kinase 5 (ERK5), which is a member of the MAPK family (112).

1.3.2.4 HSP90

Most studies regarding heat shock proteins (HSPs) in the context of ALK pathobiology have been focused on HSP90, which is a molecular chaperone that plays a crucial role in helping proteins, called clients, fold correctly (59). Many ALK fusion proteins, particularly EML4-ALK and NPM-

ALK, interact with and are shown to be clients of HSP90 (59, 113). Many HSP90 inhibitors showed substantial efficacy in ALK^+ cancers (59, 114, 115). For instance, NVP-AUY922 and IPI-504 showed strong antitumor effect in ALK-driven NSCLC (116, 117). Another study showed that crizotinib-resistant tumor cells remained addicted to ALK signaling but retained sensitivity to the HSP90 inhibitor 17-AAG (tanespimycin) (114).

1.3.2.5 Src

Src is a non-receptor tyrosine kinase that plays an important role in cancer progression (118). NPM-ALK was shown to interact with and to activate the Src kinase pp60 or c-src (67). Phosphorylation at tyrosine residue 418 of NPM-ALK was shown to be required for activating Src-kinases (67). The interaction between Src and NPM-ALK is also important for mediating NPM-ALK oncogenesis as inhibition of Src or loss of this interaction results in downregulation of NPM-ALK-mediated cell proliferation (67). In one study, Src inhibition using inhibitors (such as saracatinib (AZD0530), dasatinib and KIN001-113 or siRNA knockdown was shown to be consistently effective in resensitizing cells to ALK inhibition in several patient-derived ALK-positive NSCLC models (carries EML4-ALK fusion) (119). Further experiments need to be performed to understand the relation between Src and other ALK forms across ALK^+ cancers.

1.3.2.6 Other ALK-interacting proteins

ShcC, a Src homology 2 domain-containing adaptor protein, was shown to form a complex with ALK, full-length and NPM-ALK, resulting in deregulation of the responsiveness of the MAPK pathway to growth factors (120-122). Inhibition of binding of ALK to ShcC significantly impairs the survival, differentiation and motility of neuroblastoma cells with activated ALK by blocking the MAPK and PI3K/AKT pathways and inducing apoptosis (120).

MSH2 (MutS homolog 2), a key DNA mismatch repair (MMR) protein integral to the suppression of tumorigenesis, was shown to be an NPM-ALK-interacting protein (76). Subsequent work identified NPM-ALK-induced phosphorylation of MSH2 at the tyrosine residue 238 as a crucial event in suppressing MMR (123).

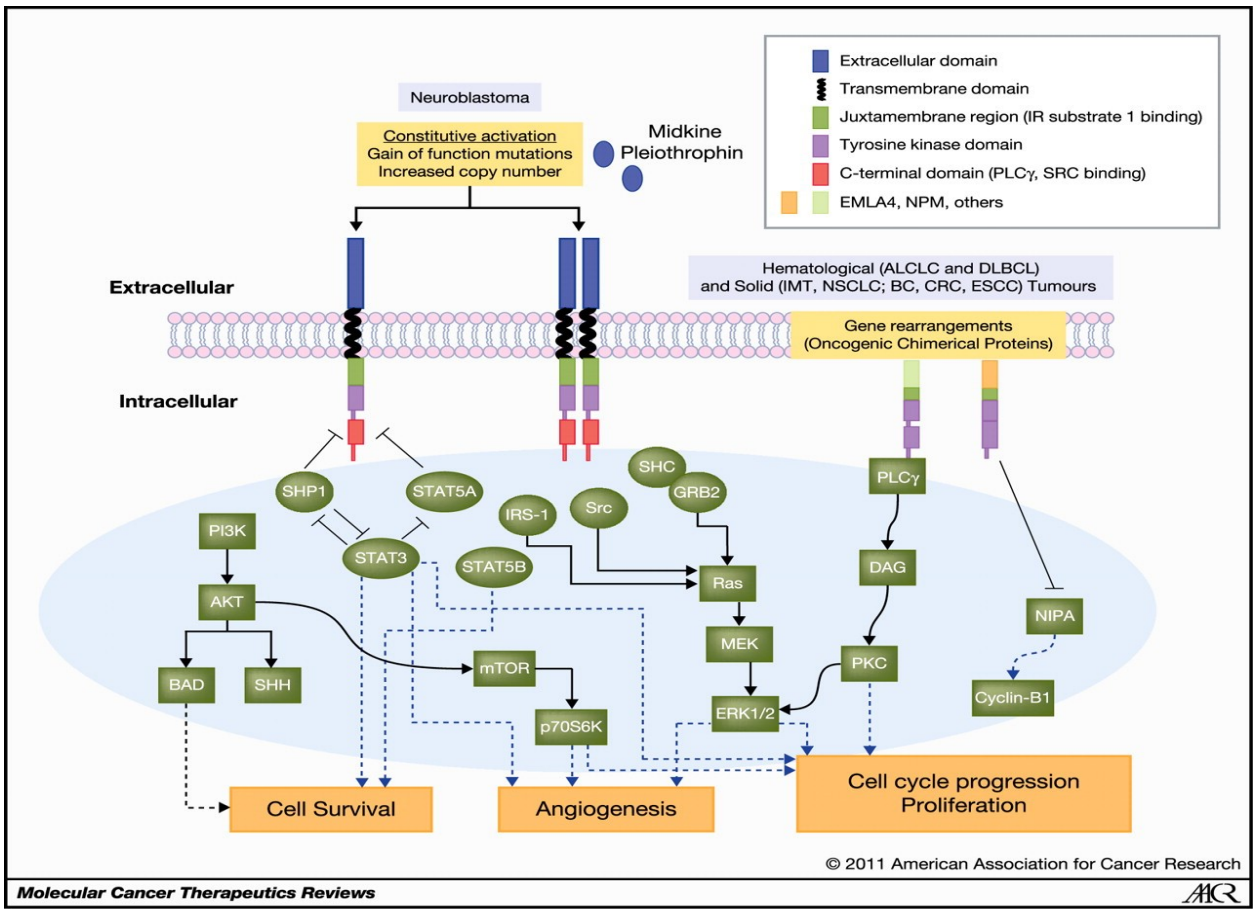

Figure 1.25 Oncogenic ALK forms and associated signaling pathways.

A schematic diagram of different ALK forms. The mechanisms associated with deregulation are in solid lines. Hematological malignancies are shown in the figure's upper part. The hypothetical downstream signaling pathways and cellular responses subsequent to ALK constitutive activation in tumors are shown in the figure's lower part. Abbreviations: PLC-γ–phospholipase C gamma; ALCL–anaplastic large cell lymphoma, conventional; DLBCL–diffuse large B cell lymphoma; IMT–inflammatory myofibroblastic tumor; NSCLC–non-small cell lung cancer; BC–breast cancer; CRC–colorectal cancer; ESCC–esophageal squamous cell carcinoma; SHP1–Src homology region 2 domain-containing phosphatase-1; STAT–signal transducer and activator of transcription; SHC–Src homology region 2 domain-containing; GRB2–growth factor receptor-bound protein 2; IRS-1–insulin receptor substrate 1; PI3K–phosphatidylinositide 3-kinase; DAG–diacylglycerol; NIPA-nuclear interaction partner of ALK; MEK–mitogen-activated protein kinase; BAD–Bcl2-associated death receptor; SHH–sonic hedgehog; mTOR–mammalian target of rapamycin; PKC–protein kinase C; ERK–extracellular signal-related kinase.
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1.4 ALK in disease: fusion proteins, overexpression and mutation

The *ALK* gene can be oncogenic either by forming a fusion gene with any of several other genes, by gene amplification, or by mutations (124). Almost all the *ALK* translocations are derived from genomic breakpoints within the intron between exon 19 and 20 of *ALK*, resulting in the fusion of the distal portion of the *ALK* gene (encoding for the intracytoplasmic domain of ALK) with the promoter region and the proximal domain of the gene encoding for the fusion partner (58, 125). *ALK* fusion partners share three main features: (i) The promoter of the gene of the ALK partner protein drives the transcription of the fusion gene; (ii) The subcellular localization of the fusion protein is determined by the ALK partner protein; (iii) The *ALK* partner is responsible for the oligomerization and the consecutive autophosphorylation of the chimera (126-128). As shown in **Table 1.1**, over 22 *ALK* different fusion proteins have been discovered in multiple cancer types (124). Amplification of the *ALK* locus, mutation of *ALK* and overexpression of ALK protein has been reported in many different types of cancer cell lines and in human tumor samples, including neuroblastoma (16). This section will cover three ALK-positive cancers; ALCL, NSCLC and neuroblastoma.

1.4.1 Anaplastic Large Cell Lymphoma (ALCL)

1.4.1.1 What is ALCL?

Stein et al characterized anaplastic large-cell lymphoma (ALCL) in 1985, by reporting a consistent expression of CD30 (previously called Ki-1 antigen) in tumors with frequent cohesive proliferation of large anaplastic (bizarre) cells (129). Subsequent studies showed that most of ALCL tumors are derived from lymphoid cells of T or null immunophenotype (59). ALCL is a relatively rare, aggressive type of non-Hodgkin lymphoma (NHL) (10). Almost all ALCL cells are characterized by the existence of so-called hallmark cells (abundant cytoplasm, kidney-shaped nuclei, and a paranuclear eosinophilic region) and CD30 (a cell membrane protein belonging to the tumor necrosis factor receptor family) (10). Clonal rearrangement of the T-cell receptor comprises over 90% of the ALCL cases (10).

Table 1.1 Recurrent chromosomal translocations involving ALK in cancer.

Disease	Chromosomal abnormalities	Fusion protein (kDa)	Partner	Frequency (%)	ALK IHC staining	Reference
ALCL	t(2;5)(p23;q35)	NPM–ALK (80)	*NPM1*	75–80	Cyto/nuclear	Morris et al. (1994), Shiota et al. (1994)
ALCL	t(2;17)(p23;q25)	ALO17–ALK (ND)	*ALO17*	<1	Cyto	Cools et al. (2002)
ALCL	t(2;3)(p23;q21)	TFG–ALK (113)	*TFG*	2	Cyto	Hernandez et al. (1999, 2002)
ALCL	t(2;X)(p32;q11–12)	MSN–ALK (125)	*MSN*	<1	Cyto	Tort et al. (2001, 2004)
ALCL	t(1;2)(q25;p23)	TPM3–ALK (104)	*TPM3*	12–18	Cyto	Lamant et al. (1999), Siebert et al. (1999)
ALCL	t(2;19)(p23;p13)	TPM4–ALK (95)	*TPM4*	<1	Cyto	Meech et al. (2001)
ALCL	inv(2)(p23;q35)	ATIC–ALK (96)	*ATIC*	2	Cyto	Ma et al. (2000), Trinei et al. (2000), Colleoni et al. (2000)
ALCL	t(2;22)(p23;q11.2)	MYH9–ALK (220)	*MYH9*	<1	Cyto	Lamant et al. (2003)
ALCL	t(2;17)(p23;q23)	CLTC1–ALK (250)	*CLTC1*	2	Cyto	Touriol et al. (2000)
DLBCL	t(2;5)(p23;q35)	NPM–ALK (80)	*NPM1*	N/A	Cyto/nuclear	Adam et al. (2003), Onciu et al. (2003)
DLBCL	t(2;17)(p23;q23)	CLTC1–ALK (250)	*CLTC1*	N/A	Granular cyto	De Paepe et al. (2003)
DLBCL	t(2;5)(p23.1;q35.3)	SQSTM1–ALK (ND)	*SQSTM1*	N/A	Cyto	Takeuchi et al. (2011)
DLBCL	ins(4)(2;4)(?;q21) t(2;4)(p24;q21)	SEC31A–ALK (ND)	*SEC31A*	N/A	Cyto	Bedwell et al. (2011), Van Roosbroeck et al. (2010)
IMT	t(1;2)(q25;p23)	TPM3–ALK (104)	*TPM3*	50	Cyto	Lawrence et al. (2000)
IMT	t(2;19)(p23;p13)	TPM4–ALK (95)	*TPM4*	<5	Cyto	Lawrence et al. (2000)
IMT	t(2;17)(p23;q23)	CTLC–ALK (250)	*CLTL*	<5	Cyto	Bridge et al. (2001), Patel et al. (2007)
IMT	inv(2)(p23;q35)	ATIC–ALK (96)	*ATIC*	<5	Cyto	Debiec-Rychter et al. (2003)
IMT	t(2;11;2) (p23;p15;q31)	CARS–ALK (ND)	*CARS*	<5	Cyto	Cools et al. (2002), Debelenko et al. (2003)
IMT	t(2;2)(p23;q13) inv(2)(p23;p15;q31)	RANBP2–ALK (ND)	*RANBP2*	<5	N/M	Ma et al. (2003), Marino-Enriquez et al. (2011)
IMT	t(2;4)(p23;q21)	SEC31L1–ALK (ND)	*SEC31L1*	<5	Cyto	Panagopoulos et al. (2006)
ESCC	t(2;19)(p23;p13)	TPM4–ALK (110)	*TPM4*	N/A	Cyto	Jazii et al. (2006), Du et al. (2007)
RCC	t(2;10)(p23;q22)	VCL–ALK (117)	*VCL*	N/A	Cyto	Debelenko et al. (2011)
NSCLC	inv(2)(p21;p23)	EML4–ALK (120)	*EML4*	2–5	Cyto	Rikova et al. (2007), Soda et al. (2007)
NSCLC	t(2;3)(p23;q21)	TFG–ALK (113)	*TFG*	2	Cyto	Rikova et al. (2007)
NSCLC	t(2;10)(p23;p11)	KIF5B–ALK (ND)	*KIF5B*	<1	Cyto	Takeuchi et al. (2009), Wong et al. (2011)
NSCLC	t(2;14)(p23;q32)	KLC1–ALK (ND)	*KCL1*	<5%	Cyto	Togashi et al. (2012)
NSCLC	t(2;9)(p23;q31)	PTPN3–ALK (ND)	*PTPN3*	N/A	Cyto	Jung et al. (2012)
CRC	inv(2)(p21;p23)	EML4–ALK (120)	*EML4*	<5%	Cyto	Lin et al. (2009)
CRC	t(2;2)(p23.3)	C2orf44–ALK (ND)	*C2orf44*	N/A	Cyto	Lipson et al. (2012)
BC	inv(2)(p21;p23)	EML4–ALK (120)	*EML4*	<5%	Cyto	Lin et al. (2009)

ND, not determined; Cyto, cytoplasmic; N/M, nuclear/membrane; N/A, non-assessed; ALCL, anaplastic large cell lymphoma, DLBCL, diffuse large B-cell lymphoma; IMT, inflammatory myofibroblastic tumor; ESCC, esophageal squamous cell cancer; RCC, renal cell cancer; NSCLC, non-small-cell lung cancer; CRC, colon–rectal cancer; BC, breast cancer.

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1.4.1.2 ALK-positive ALCL

ALK^+ALCL, in the form of NPM-ALK fusion, accounts for more than 90% of ALCL cases in children and adolescents, which account for ~15% of all NHL cases (130). On the other hand, ALK^+ALCL accounts for ~50% of adult ALCL patients, which accounts for 2% of adult NHL (131). Additionally, the median age for patients diagnosed with ALK^+ALCL is around 30 years while it is 55 years in ALK^-ALCL (132). NPM-ALK chimeric protein, which is derived from t(2;5)(p23;q25) translocation, can be detected in 80% of ALK^+ALCL (126). Very few studies have focused on other ALK fusion proteins in ALK^+ALCL such as TPM3-ALK and TFG-ALK fusion proteins (131).

ALK^+ALCL have a better prognosis in comparison to ALK^-ALCL (132). The most commonly chemotherapeutic regimens applied for ALK^+ALCL include Cyclophosphamide, hydroxydaunorubicin (doxorubicin), oncovin (vincristine), and prednisone (CHOP) (131). While cure rate can reach up to 80%, relapses and resistance occur in many cases (133, 134). Patients with relapsed or refractory disease usually benefit from high-dose chemotherapy or autologous stem cell transplantation (132). However, some of these relapsed cases might need more specific therapeutic targeting. For instance, Brentuximab vedotin has showen encouraging clinical outcomes when it was applied to treat ALK^+ALCL and other $CD30^+$ peripheral T-cell lymphomas (135).

1.4.2 Non-small cell lung cancer (NSCLC)

1.4.2.1 What is NSCLC?

Non-small cell lung cancer (NSCLC) accounts for 85–90% of lung cancers, which is the leading cause of cancer-related death worldwide, and has an average of a five-year survival rate of only 15% (136). Comprehensive molecular profiling of this cancer type identified several tyrosine kinase drivers, including the *epidermal growth factor receptor (EGFR)* gene and the echinoderm microtubule-associated protein-like 4 - anaplastic lymphoma kinase (*EML4-ALK*) gene

rearrangement (137). The EML4-ALK fusion oncogene, which arises from an inversion on the short arm of chromosome 2 (Inv(2)(p21p23) that joins exons 1 to 13 of *EML4* and exons 20-29 of *ALK*, was first demonstrated in resected lung adenocarcinoma specimens (49). Subsequent studies have estimated that 5% to 8% of NSCLCs harbor an EML4-ALK fusion protein (10, 138). It is interesting to note that EML4-ALK$^+$NSCLC patients are mainly never or light smokers, and they are younger than the EML4-ALK$^-$NSCLC group, with a median age of 52 years compared to 64 years, respectively (139).

NSCLC patients are very insensitive to chemotherapy and radiation, thus making surgery the treatment of choice for this type of cancer. However, only a small proportion of NSCLCs are diagnosed before tumor cells metastasize. Paclitaxel, which stabilizes microtubules and interferes with their breakdown during cell division, and carboplatin, which interferes with DNA repair, are the two commonly used chemotherapeutic regimes (140). In recent years, targeting oncogenic drivers has transformed the care of patients with advanced lung cancer (10). Erlotinib (Tarceva) and Gefitinib (Iressa) are EGFR inhibitors that are prescribed as second-line treatment of advanced NSCLCs (10).

1.4.2.2 ALK in NSCLC

Although the proportion of NSCLCs with the EML4-ALK fusion proteins is low (5% to 8%), the total number of cases of ALK$^+$NSCLC amenable to treatment with ALK inhibitors is greater than that for all other known ALK-related cancers combined (10). Early trials using crizotinib, an ALK inhibitor, showed remarkable response rates and prolonged progression-free survival (PFS) compared with chemotherapy in ALK$^+$NSCLC patients in both pretreated and first-line settings (141, 142). Not unexpectedly, almost every ALK-rearranged tumor eventually acquires resistance during crizotinib treatment within one year since the initiation of treatment (141, 143). Second- and third-generation ALK inhibitors have been developed to overcome acquired crizotinib resistance (143). For instance, ceritinib (LDK378) and alectinib (CH5424802/RO5424802) are already approved by U.S. Food and Drug Administration (FDA) in crizotinib-resistant patients (143). Other inhibitors such as brigatinib (AP26113), AZD3463, lorlatinib (PF-06463922) and entrectinib (RXDX-101) are in different phases of clinical development (143).

1.4.3 Neuroblastoma (NB)

1.4.3.1 What is NB?

Neuroblastoma (NB) is a neuroendocrine tumor arising from progenitor cells of the neural crest element of the sympathetic nervous system (144). NB has been considered as a developmental disorder since it develops through aberrant regulation of signaling pathways that governing developmental lineage commitment and differentiation of the sympathetic nervous system (144). NB is the most common extracranial solid tumor childhood malignancy, accounting for 7% of childhood malignancies and 15% of childhood cancer mortality (10, 145).

1.4.3.2 Clinical features

While NB is mostly very aggressive, the clinical presentation can be highly variable and some tumors cause no illness (146). The incidence of NB has increased recently and it continues to carry a poor prognosis in children over 18 months of age with a survival of only 38% (145). As shown in **Table 1.2**, NB is risk-stratified based on disease stage, age and biological features (i.e. *MYCN* status, tumor histology and DNA ploidy) (147), with survival ~90% for low and intermediate risk and ~40% for high-risk group (148, 149).

Table 1.2 Neuroblastoma risk groups

Risk group	Stage	Age	*MYCN* Amplification Status	Ploidy	Shimada
Low risk	1	Any	Any	Any	Any
Low risk	2a/2b	Any	Not amplified	Any	Any
High risk	2a/2b	Any	Amplified	Any	Any
Intermediate risk	3	<547 d	Not amplified	Any	any
Intermediate risk	3	≥547 d	Not amplified	Any	FH
High risk	3	Any	Amplified	Any	Any
High risk	3	≥547 d	Not amplified	Any	UH
High risk	4	<365 d	Amplified	Any	Any
Intermediate risk	4	<365 d	Not amp	Any	Any
High risk	4	365 to <547 d	Amplified	Any	Any
High Risk	4	365 to <547 d	Any	DI = 1	Any
High risk	4	365 to <547 d	Any	Any	UH
Intermediate risk	4	365 to <547 d	Not amplified	DI > 1	FH
High risk	4	≥547 d	Any	Any	Any
Low risk	4s	<365 d	Not amplified	DI > 1	FH
Intermediate risk	4s	<365 d	Not amplified	DI = 1	Any
Intermediate risk	4s	<365 d	Not amplified	Any	UH
High risk	4s	<365 d	Amplified	Any	Any

Abbreviations: DI, DNA index; FH, favorable histology; UH, unfavorable histology.
Courtesy of Children's Oncology Group.

1.4.3.3 Key biological defects

Multiple signaling pathways, including transcription factors, are involved in NB pathogenesis (145). These pathways exhibit a wide array of biological characteristics and behaviors, with some of them being important in predicting outcomes (145). For example, *MYCN* amplification was shown to regulate multiple crucial cellular processes in NB cells such as cell cycle, apoptosis and differentiation (150). In addition to *MYCN*, two other oncogenes, *ALK* and *LIN28B*, were found to be amplified (150).

The amplification of *MYCN*, which is a phosphoprotein in the MYC family of transcription factors encoded by the *MYCN* gene, was detected in 20% to 25% of NB tumors and was shown to be a biomarker for high-risk neuroblastomas (151). N-MYC functions as a transcription factor through promotor binding and association of activator or repressor protein complexes (151). For instance, N-MYC binds to a histone methyl transferase enzyme called enhancer of zeste homolog 2 (EZH2) and thereby it plays a key role in N-MYC-controlled gene repression of neuronal differentiation genes such as *clusterin* (152). Additionally, N-MYC has been shown to increase cell proliferation in NB cells by inducing the expression level of many of its transcription targets, such as the transient receptor potential cation channels M6 and M7 (TRPM6 and TRPM7) (153) and the high mobility group A1 (HMGA1) (154). There are a number of the drugs targeting N-MYC that are already in early phase clinical testing and others that are at an advanced stage of pre-clinical evaluation (145, 155).

1.4.3.4 ALK in NB

1.4.3.4.1 Identification

Since 2000, the expression of full-length ALK, as mRNA and protein, has been detected in almost all primary NB and NB cell lines (156-158). However, the identification of familial and sporadic *ALK* mutations and its critical role in the pathogenesis of NB that challenged the NB field came in 2008 because this represented an important novel druggable target (51-54). *ALK* mutations account for 8.4% and *ALK* amplification accounts for 3.5% of NB patients (54). The initial report identified 11 mutations in which 3 were familial, 9 were sporadic, and one was familial and sporadic (54).

Subsequent reports identified that 50% of familial NB has dominant mutations in *ALK*, with R1275Q being the most frequent (51, 54). Two hotspot mutations, R1275 and F1174, were reported in 85% of NB patients; the R1275R mutation was found in both familial and sporadic while F1174L mutation was restricted to sporadic tumors (40). A very recent report identified a novel *ALK* fusion (*BEND5–ALK*) in one out of 230 screened NB patients, however, its oncogenic properties are still unknown (159). Therefore, ALK in NB demonstrates a wide range of genetic aberrations.

1.4.3.4.2 Biological significance

Most of ALK studies in NB focused on the role of *ALK* mutants. For example, ALK^{F1174L} was shown to possess a gain-of-function kinase activity that can sustain important signaling pathways, such as Stat3 and Akt, in Ba/F3 cells cultured in the absence of IL-3 (52). Another study showed that mutant ALK, either ALK^{F1174L} or ALK^{R1275Q}, drives upregulation of MAPK negative feedback regulators and upregulates RET and RET-driven sympathetic neuronal markers (160). Enzymatic analysis of a purified catalytic domain of ALK^{F1174L} and ALK^{R1275Q} exhibits ~ 4-fold and ~ 8-fold higher catalytic efficiency, respectively, compared to the wild-type ALK (161).

A few studies provide evidence for molecular connections between ALK and N-MYC through multiple mechanisms. For example, ALK was shown to drive N-MYC expression by promoter activation and activation through ERK5. Specifically, one study showed that ALK^{wt} and the two activating mutations (ALK^{R1275Q} and ALK^{F1174L}) upregulate endogenous *MYC* transcript level (162). Another study showed that ALK stimulates ERK5 to promote the expression of the oncogene N-MYC in NB cells (112). Furthermore, ALK^{F1174L} was shown to activate downstream signaling through PI3K/AKT and, thereby controlling GSK-3β activity and N-MYC protein stabilization (163). Other studies documented an accelerated tumor formation in N-MYC transgenic mice and zebrafish upon the expression of ALK^{F1174L} (164, 165).

Prognostically, ALK^{F1174L} was shown to be preferentially associated with *MYCN* gene amplification in NB, which defines a subset of ultra-high-risk NB patients with distinctly poor outcome (166). In addition, ALK^{F1174L} represents one of the sites of secondary kinase domain mutations in cases of ALK-rearranged cancers (167). For example, this mutation inhibits

crizotinib-mediated downregulation of ALK signaling and blocks apoptosis when introduced into *RANBP2-ALK* expression plasmid (167).

1.4.3.4.4 Detection methods

Previous studies commonly used two techniques for ALK detection in NB, which are immunohistochemistry (IHC) and reverse-transcriptase polymerase chain reaction (RT-PCR). There is no standard protocol for IHC as some ganglion cells expresses ALK. Common antibodies that are used are 5A4 (Novocastra, Newcastle, UK), D5F3 (Cell signaling technology, Danvers, MA, USA) and ALK clone ZAL4 (Invitrogen, Carlsbad, CA, USA) (summarized in **Table 1.3**). There are advantages to IHC such as low cost, easy and less time consuming. However, challenges in using IHC include: 1) tissue preparation, 2) choice of antibody, and 3) signal enhancement systems. By IHC, ALK expression level was shown to be associated with short survival in NB patients (166, 168-170). It is important to point out that, despite the fact that different anti-ALK antibodies were employed in each of the 4 studies shown in **Table 1.3**, and that the scoring criteria used in these studies were not uniform, the prognostic significance of ALK immunostaining scores was identified in these studies. Importantly, all four studies used a cut-off of 50% ALK positive immunostained tumor cells.

For RT-PCR, early studies identified ALK to be positive in >90% of NB patients (156). However, RT-PCR is a very sensitive assay and contamination of tumor cells with ganglionic cells might be the cause of false-positive read-outs in some cases. Thus, it is very challenging to interpret the RT-PCR results apart from IHC results.

Table 1.3 Respective levels of ALK expression in four independent neuroblastoma cohorts by IHC.*

Antibody	Scoring criteria	Prognostic significance	Ref.
Anti-ALK mouse antibody Ab-1 (Clone 5A4)	• 1+, <10% reactive neoplastic cells; • 2+, between 20% and 50% reactive neoplastic cells, heterogeneous with low-moderate intensity of staining; • 3+, >50% reactive neoplastic cells, with heterogeneous moderate/high intensity of staining; • 4+, >75% reactive neoplastic cells with strong intensity of staining.	The 3-year EFS probability for patients with: - absent/low (0, 1+) ALK positivity was 0.87 ± 9 - high (3+, 4+) ALK positivity was 0.32 ± 7 - *P* <0.001.	(169)
The α-ALK/p80 monoclonal antibody (RM-9108)	• category 1, <20% ALK-positive cells; • category 2, 20% to 50% ALK-positive cells; • category 3, 50% to 75% ALK-positive cells; • category 4, 75% to 100% ALK-positive cells.	Higher ALK positivity correlates with inferior overall survival (*p*=0.002).	(168)
ALK polyclonal antibody (NBP1-00711)	• ALK was considered positive when more than 50% of tumor cells were stained as previously reported in (169) and (166).	The 3-year overall survival rate of patients with ALK negative was 70.0%, which was significantly higher than the 33.3% rate in patients with ALK positive (*p*= 0.044)	(170)
Anti-ALK (clone ALK1, Dako)	• 0 meant no or weak staining intensity (<10% of the cells), • 1 meant weak staining intensity (10-50% of the cells), • 2 meant medium staining intensity (50-80% of the cells), • 3 meant high staining intensity (>80% of the cells).	ALK protein expression ≥2 shows a significant correlation with overall survival (OS; *p*= 0.014) and progression-free survival (PFS; *p*= 0.002)	(166)

* This table summarizes the prognostic significance of ALK immunostaining scores in 4 studies.

1.4.4 ALK in other cancers

1.4.4.1 Inflammatory myofibroblastic tumor (IMT)

Inflammatory myofibroblastic tumor (IMT) is a rare mesenchymal neoplasm which occurs in soft tissues, usually during the first two decades of life (171). IMTs typically arise in the lung, abdomen, or pelvis; it is preferentially treated by surgical excision (10). However, local recurrence may occur after surgery (10). Post-surgery chemotherapeutic regime consists of cisplatin, doxorubicin (a DNA intercalator), and mitomycin C (a DNA crosslinker) (171). About 50% of IMTs possess ALK rearrangements, such as TPM3-ALK fusion, which is considered as a favorable prognostic factor (172, 173).

1.4.4.2 Rhabdomyosarcoma (RMS)

Rhabdomyosarcoma (RMS) is another rare mesenchymal neoplasm which commonly occurs in soft tissues, particularly skeletal muscle, of children and adolescents and accounts for approximately 250 cases of childhood cancer in the United States each year with a five-year survival rate of 50–85% (174). RMS tumors are classified histologically as embryonal, alveolar, and pleomorphic (10). Embryonal RMS accounts for two-third of all cases, which is followed by the alveolar and then the rare pleomorphic subtype (10). RMS typically arises in the head, neck, bladder, arms, legs, and trunk (175). RMS treatment protocols developed by the Soft Tissue Sarcoma Committee of the children's Oncology Group include surgery, radiotherapy, and chemotherapy (10). Standard therapeutic regimens consist of a combination of oncovin (vincristine), actinomycin D (a potent transcription inhibitor), and cyclophosphamide (a potent translation inhibitor) (175). The ALK protein, as a full-length form, was shown to be highly expressed in alveolar RMS and less so in the embryonal subtype (81% versus 32%, respectively) (176). ALK expression also correlated with a poor prognosis and metastasis in this type of cancer (177).

1.4.4.3 Breast Cancer

Further ALK aberrations were also identified in several other cancer types (10, 57), however, the oncogenic impact of these aberrations were not being validated. For example, ALK in breast cancer was reported using IHC and claimed to be expressed in all 63 breast cancer sections analyzed (178). Another report, which used exon array profiling, found *EML4-ALK* fusion in 2 breast cancer cell lines (HCC1500 and ZR75-1) out of 84 screened breast cancer cell lines (179). In a recent study, *Robertson et al* (2013) analyzed 25 inflammatory breast cancer (IBC) patient tumors using the FDA approved diagnostic test for *ALK* genetic abnormalities and found that 80% had increased *ALK* copy number and one patient was identified as having an *EML4-ALK* translocation (180). Additionally, crizotinib treatment induced both cytotoxicity (IC_{50} = 0.89 μM) and apoptosis, with abrogation of pALK signaling in tumor cells (180).

1.4.4.4 Esophageal Cancer

In esophageal cancer, *Jazii et al* (2006) published the first report describing the expression of the TPM4–ALK fusion protein in squamous cell carcinomas (SCCs) of the esophagus in a population of Iranian patients (181). *Du et al* (2007) found that TPM4-ALK fusion oncoprotein type 2 was one out of 22 proteins differentially expressed between esophageal squamous cell carcinomas (ESCCs) and normal esophageal tissues using proteomic profiling of proteins (182). In neither of these reports was the expression of TPM4–ALK independently confirmed using other methodologies (e.g., anti-ALK immunostaining, RT-PCR and/or FISH to identify the t(2;19)(p23;p13) chromosomal translocation that generates this *ALK* fusion gene), nor was the incidence of TPM4–ALK-expressing tumors carefully examined. In 2013, *Schoppmann et al* found that *ALK* amplification, but not translocation, is a frequent event in esophageal cancer and accounts for 10% of gene amplification using FISH (183). Another group looked at exome and whole-genome sequencing of esophageal adenocarcinoma and revealed multiple *ALK* mutations (one insertion, one deletion, two silent and 6 missense mutations) in 10 out of 145 screened patient samples (184).

1.5 ALK targeting using tyrosine kinase inhibitors (TKIs)

1.5.1 Crizotinib

Crizotinib was the first ALK inhibitor treatment to enter clinical trials (185). It binds selectively to the inactive conformation of both MET and ALK and has shown striking efficiency against ALK-positive tumors, both in mouse models and in the clinical trials (40). Crizotinib represents a breakthrough treatment for some ALK^+ NSCLC patients, and there is an increased urgency for it to be approved for many other ALK^+ cancer patients (186). Quite remarkably, the Children's Oncology Group phase 1 trial of single-agent crizotinib revealed marked antitumor activity in eight out of nine patients with relapsed ALK^+ALCL at a range of doses (187). A similar robust effect was observed in two relapsed ALK^+ALCL patients (188). Furthermore, two randomized trials have shown that crizotinib is superior to standard chemotherapy in patients with advanced/metastatic ALK^+ NSCLC (141, 142).

1.5.1.1 Differential responsiveness to crizotinib treatment

The differential responsiveness to crizotinib treatment among different ALK^+ cancer patients represent a current problem and a scanty number of studies has reported that (24, 56). As shown in **Table 1.4**, I have summarized data extracted from publications that have applied different ALK inhibitors into ALK^+ cell lines and has shown a wide range of IC_{50}, even amongst cells that carry the same ALK form. In order to understand the reason behind the differential crizotinib responsiveness, one study showed that the high sensitivity of a cell line that harbors $ALK^{amplification}$, NB1, while cell lines that harbor ALK mutations or wild-type ALK were very insensitive when treated with crizotinib (189). This study stated that mutated ALK (specifically at the site F1174L) has high ATP affinity, which is the reason why crizotinib was not affecting those cells, however, increasing the crizotinib concentration would overcome this insensitivity (189). In contrast, *Moore et al* demonstrated that the crizotinib IC_{50} of two cell lines that harbor the same mutation (namely ALK^{F1174L}) were 5-fold different (IC_{50} for Kelly cells was 400 nM while for LAN-1 cells was 2000 nM) (190). These variable results suggest that the form of ALK or ALK mutation site is not the only determinant of its responsiveness to crizotinib treatment.

Among different ALK^+ cancer types, there is a wide range of responsiveness to crizotinib treatment with largely unknown mechanisms (9, 56). Additionally, resistance to crizotinib invariably develops mostly within a year (191). In the NSCLC model, the crizotinib differential sensitivity in EML4-ALK-expressing cells was justified using four constructs that were corresponding to four EML4-ALK variants, where the differences in protein stability were dependent on the EML4-ALK variant (24). However, subsequent clinical trials failed to correlate EML4-ALK variants with crizotinib responses in ALK^+ tumors (192). One study observed an overall response rate of 57% (47 out of the 82 patients, with 46 confirmed partial responses and one complete response) (193). Another study that included 53 patients had one complete response, 31 partial responses and 13 stable diseases (194). A third study that included 82 patients, showed no complete response, 57 (69.5%) partial response, 15 (18.3%) stable disease and 10 (12.2%) with progressive disease demonstrating no obvious correlation between particular *ALK* variant and objective response rate (ORR) or progression-free survival (PFS) during crizotinib treatment (195).

In a neuroblastoma model, a wide range of crizotinib responsiveness was found in phase 1 clinical trial of ALK^+ neuroblastoma patients (187). Forty-three patients were treated with crizotinib in which 11 of them were with known *ALK* mutations (four F1174, one F1245, five R1275 and one Y1278) and the other 23 patients with unknown *ALK* status. Only 2 out of the 34 patients showed complete remission (6%) and 8 exhibited a stable disease (23.5%). However, the majority of them (24 out of 34 patients) exhibited a progressive disease despite intensive crizotinib treatment (70.5%) (187). An ongoing phase 2 trial of crizotinib has shown activity in a subset of neuroblastoma patients, but the frequency and duration of responses were marginal (186, 196). More recently, combining crizotinib with conventional genotoxic agents was suggested as a strategy and may provide superior antitumor activity than when either approach is used alone (196). Therefore, more studies are needed to provide explanation for the observed differential crizotinib responsiveness.

Table 1.4 ALK inhibitors show wide range of IC_{50} among different ALK-positive cancer cells. *

Cancer type	Cell line	Inhibitor	ALK form	IC_{50} (nM)	Reference
ALCL	Karpas 299	Crizotinib	*NPM-ALK*	32	(197)
	SU-DHL-1		*NPM-ALK*	43	
NSCLC	H2228	Crizotinib	*EML4-ALK v3*	871	(198)
	H3122		*EML4-ALK v1*	1551	
Neuroblastoma	SH-SY5Y	TAE-684	Full-length ALK^{F1174L}	258	(52)
	KELLY			416	
	SK-N-SH			0.529	
	SMS-KCNR		Full-length ALK^{R1275Q}	4932	
	IMR32		Full-length $ALK^{wild\text{-}type}$	1663	
	IMR5			1789	
	NGP			1708	
	CHP-100			2106	
	BE(2)M17			736	
	BE(2)C			743	
Neuroblastoma	SK-N-SH	TAE-684	Full-length ALK^{F1174L}	9	(199)
	AMC106c			67	
	KCNR			28	
	LAN-5		Full-length ALK^{R1275Q}	9	
	SK-N-BE		Full-length $ALK^{amplification}$	349	
	NMB			591	
	SJNB10		Full-length $ALK^{wild\text{-}type}$	240	
	SJNB12			268	
	SJNB1			283	
	SK-N-AS			497	
	SJNB6			553	
	SJNB8			588	

* This table shows data extracted from publications that have applied different ALK inhibitors into ALK^{+} cell lines.

1.5.1.2 Crizotinib secondary resistance

Initial reports of the promising therapeutic potential of crizotinib for the treatment of ALK^{+}NSCLC patients was, unfortunately, shortly accompanied by the identification of many secondary mutations, such as G1202R and G1269A, in the ALK kinase domain (16). These ALK mutations were shown to account for approximately 30% of the failures to crizotinib treatment (200). There are additional alternative methods of crizotinib resistance. These include ALK amplification, aberrant amplification of KIT, and mutation of KRAS and EGFR, which have all been identified in patient samples (201, 202). These alterations bypass the requirement for ALK activity in the tumor cells. Thus, crizotinib resistance is a complex problem that includes ALK kinase domain mutations, copy number gain of ALK and/or other oncogenes.

1.5.1.3 Approaches to overcome crizotinib resistance

Patients under crizotinib therapy are considered resistant when an increase in size or in number of lesions is observed (203). Furthermore, not all cancer progressions necessarily require an immediate therapeutic change as some patients may progress slowly, in limited pre-existing sites or in a single new site, and without deterioration of their symptoms (204). In these cases, premature discontinuation of crizotinib therapy would not be the preferred therapeutic choice due to the risk of a rapid disease progression (i.e. disease flare) (204).

The current approach to overcome crizotinib resistance mainly relies on second and third generation's TKIs with over 11 inhibitors being developed (205, 206). However, preclinical studies reported that these inhibitors were sensitive to some mutations but resistant to others (207-209). For example, one study showed that Ceritinib could overcome Alectinib resistance (207). In contrast, other studies demonstrated that Alectinib could overcome the resistance induced by L1196M and G1269A mutations, which occurred in patients with Ceritinib-resistance (208, 209). These results clearly demonstrated the complexity of this problem, as the efficacy of these new inhibitors is neither consistent nor predictable.

As we gain more experience about TKI resistance, we have seen very disappointing clinical trials of agents targeting other tyrosine kinases (206). For instance, third-generation EGFR TKIs are

being developed as part of the strategy to overcome treatment resistance to first- and second-generation EGFR TKIs in lung cancer patients (210). Similarly, despite the clinical efficacy of the first-, second- and third-generation BCR–ABL inhibitors, resistance occurs invariably and more than 50 distinct point mutations encoding single amino-acid substitutions in the kinase domain of the *BCR–ABL1* gene have been detected in patients with imatinib-resistant chronic myeloid leukemia (CML) (211, 212). Therefore, it is crucial to identify new strategies to overcome the TKI resistance that consistently occurrs in ALK^+ cancer patients upon treatment with crizotinib.

1.5.1.4 Current approaches used to measure the effectiveness of crizotinib and other kinase inhibitors targeted therapy

The current readout used to measure the effect of ALK inhibitors focuses on phenotypic assays in which the response to an inhibitor is based on a functional readout, such as changes in the phosphorylation status of downstream targets or impact on cellular viability (58). For example, the kinase assay was used for decades to measure the effect of TKIs on the phosphorylation of selected protein kinases or specific tyrosine residues, which was assessed by a sandwich ELISA method using specific capture antibodies (213). Another readout can be achieved by measuring the effect of tyrosine kinase inhibitors on a functional property of cancer cells, such as the effect on cell proliferation (197). Additionally, the effect of TKIs can be achieved by looking into its effect on tumor xenografts and measuring the antitumor efficacy and the tumor regression upon TKI administration (197).

Previous studies evaluated the efficacy of crizotinib in inhibiting tumor cell growth across a panel of >150 cell lines derived from diverse tumor types (185, 197). These studies have indicated that the crizotinib growth-inhibitory properties at pharmacologically relevant concentrations (defined as <300 nM) are restricted to cell lines exhibiting c-Met or ALK. At concentrations >300 nM, crizotinib was shown to exhibit some inhibitory activity against related receptor tyrosine kinases, including RON, Axl, and TrkA and TrkB (185). In the rhabdomyosarcoma model, crizotinib was shown to induce antitumor activity independent on ALK and MET inhibition (214). In thyroid cancer model, non-MET related effects of crizotinib were reported to contribute to the cell-cycle arrest and cytotoxicity observed (215). It has also been reported that crizotinib has the ability to

sensitize cells to chemotherapeutic treatment in ALK-negative and MET-negative cancer cells (216). Therefore, crizotinib appears to have multiple outcomes depending on the tumor cell type or treatment conditions, such as dose-concentrations, and may have unidentified off-target antitumor effects in ALK-carrying cancers. This concern and the accumulating evidence underlines the need for further detailed investigation of different ALK forms to optimize the clinical application of crizotinib and to predict patients likely to benefit from crizotinib treatment.

1.5.2 Other ALK TKIs

1.5.2.1 Ceritinib (LDK-378)

Ceritinib is a next generation ALK inhibitor that is also known as LDK-378 or Zykadia (by Novartis) (155). In 2014, the FDA approved the use of ceritinib for treatment of ALK-positive NSCLC patients that have shown resistance to crizotinib treatment. Ceritinib, like crizotinib is an ATP competitive inhibitor that binds to the ATP binding pocket (217). Ceritinib was shown to be effective against crizotinib naïve and crizotinib treated patients (218). In a preclinical study, ceritinib was able to inhibit ALK-crizotinib resistant mutations (L1196M, I1171T, S1206Y and G1269A) but was ineffective against G1202R and F1174C mutations (217). Another study showed that ceritinib was able to overcome resistance to alectinib, another second generation ALK inhibitor, in tumors with secondary mutations I1171T and V1180L (207). Conversely, there has been a report suggesting that alectinib can overcome ceritinib resistance induced by L1196M and G1269A mutations (209).

1.5.2.2 Alectinib (CH5424802)

Alectinib is another ATP-competitive ALK inhibitor that is also known as CH5424802 (by Roche) (208). It received breakthrough therapy designation by the FDA in 2013 and was approved in Japan in 2014 (219). Alectinib has shown potent activity against many secondary ALK mutations such as L1196M, 1151ins, L1152R, C1156Y, F1174L and G1269A, however, it shows low potency in the case of the G1202R mutation (209, 220). Alectinib has shown remarkable efficacy in

intracranial metastases in crizotinib refractory patients (221). As for other inhibitors, secondary mutations were detected in tumors (I1171T and V1180L) (207).

1.6 Cancer stem cells and drug resistance

The paradigm of conventional cancer treatments, including targeted therapy, hinged on the eradication of a presumably biologically uniform cancer cell population (222). The concept of cancer stem cells (CSCs), which account for intra-tumoral heterogeneity, was first demonstrated by John Dick and his team in acute myeloid leukemia (AML) in 1994 (223). They have described a cell population, $CD34^{+}CD38^{-}$, as a hematopoietic stem cell-like AML which was able to establish AML in secondary SCID mouse recipients while the bulk of AML cells that carry other immuno-phenotypes were much less efficient (223). This work was followed by tremendous studies that looked into intra-tumoral cell heterogeneity in other cancer types, implicating tumor cell subsets with stem cell features similar to the cell populations responsible for tumor initiation, progression, resistance and relapse (224-227). For example, studies on breast cancer patients identified a strong correlation between enhanced tumorigenic capacity, decreased therapeutic response, or advanced disease stage and the abundance of breast tumor cells expressing established cancer stem cell markers, such as CD44 (228, 229).

CSCs have long been speculated to be an important mechanism of drug resistance (230). It was hypothesized that CSCs retain the characteristic of being more drug resistant, frequently by exhibiting or up-regulating other components of the same targeted signaling pathways or other signaling pathways (230, 231). Thus, chemotherapies and targeted therapies may have reduced the proliferation of the bulk tumor revealing these small cell subsets (228). For instance, although EGFR tyrosine kinase inhibitors (TKIs) are targeted therapies with great potential in many aggressive cancer types, it has been demonstrated that β_3 integrin conferred resistance to EGFR TKIs in CSCs derived from TNBC, lung, and pancreatic tumor cells (232, 233). Moreover, inhibition of downstream β_3 integrin-KRAS signaling reversed the EGFR inhibitors resistance (233), implicating a potential direct link between CSCs and drug resistance. In another study, resistance to imatinib, a TKI against BCR-ABL fusion protein, was noted in CML and was associated with an increased resistance in leukemic CSCs (234). Some reports documented that

the increased expression and activity of BCR-ABL seen in a CML stem cell-enriched population was shown to contribute to their relative insensitivity to imatinib (235). Additional mechanisms that would be expected to contribute to the CSCs relative insensitivity to imatinib include low expression of the human organic cation transporter 1 (OCT1), which causes low imatinib uptake, and high expression of ABCB1, which causes high imatinib efflux, in a CML stem cell-enriched population (236). These data highlight the importance of considering the critical role of CSCs in mediating TKI-resistant phenotype.

The existence of CSCs has been demonstrated in various types of ALK-expressing cancers (237, 238). In neuroblastoma, it has been recently reported that CSC-like cells can be identified based on their expression of a number of markers, such as CD133 and nestin (237). Thus, an improved understanding of tumor heterogeneity can heavily impact more successful treatments of tumors with varying cellular CSC compositions.

As mentioned earlier, acquired resistance to crizotinib, which is caused by post-treatment changes such as alteration in drug targets and activation of compensatory survival signaling pathways, is under intensive investigations (16). However, there are no studies on the intrinsic resistance to crizotinib in ALK^+ cancers. Particularly, the correlation between the intrinsic crizotinib resistance and CSC-like cells in ALK^+ cancers has not been appreciably studied.

1.7 Cellular Thermal Shift Assay (CETSA) as tool to study drug-target engagement

A prerequisite for studying differential drug efficiency is the detection of drug-target engagement (239). The cellular thermal shift assay (CETSA) is a method which allows the study of target engagement of drug candidates in a cellular context (240-242). Importantly, this means no modifications are introduced in the drug candidates, nor are any tracers required to investigate target engagement (242). The CETSA methodology was optimized originally based on the thermodynamic stabilization and melting temperature shift assays (TSAs) observed for purified proteins as a result of ligand binding (243, 244). For more than a decade, TSAs have been of great importance in the field of drug discovery allowing compound libraries to be screened for the presence of stabilizing ligands on isolated targets using different techniques such light scattering- or fluorescence-based techniques (245-247). Although CETSA greatly broadens the utility of

TSAs, the CETSA approach builds on the same principle as conventional TSAs and the ligand-induced stabilization where the target level in more complex environments such as in cell lysates, intact cells and tissues, is investigated (240).

The assay involves treating cells with a compound of interest, heating to denature and precipitate proteins, cell lysis, and the separation of cell debris and aggregates from the soluble protein fraction (240). Whereas unbound proteins denature and precipitate at elevated temperatures, inhibitor-bound proteins remain in solution (241). CETSA, therefore, potentially has widespread applications specifically for validation of clinical drug candidates and estimation of drug efficacy in patients (248-253).

There are two studies where CETSA was used for crizotinib treatment, however, neither were performed on ALK^+ cancer type (248, 254). The first study was assessing the photosensitivity side effect of many kinase inhibitors including crizotinib, as an ALK inhibitor, on K562 (a BCR-ABL^+ CML cell line), by combining the CETSA method with multiplexed quantitative mass spectrometry (MS) (254). In the second report, the authors demonstrated a binding between crizotinib and MTH1, concluding that crizotinib works as a suppressor of mutT homologue (MTH1) activity, nucleotide pool sanitizing enzyme (248). Importantly, the crizotinib used was not the clinically used (R)-enantiomer of the drug, however, it was the (S)-enantiomer. Therefore, no study has yet reported the crizotinib—ALK binding in ALK^+ cancer cells.

Since it has been published in 2013, CETSA has become very popular as a tool to validate drug-target interactions (255, 256). For example, the proposed PARP-1 inhibitor iniparib reached phase III clinical trials, where it showed no efficacy, and was subsequently shown to lack activity against PARP-1 in living cells (255, 256). CETSA was used to compare the target engagement of PARP-1 for iniparib and olaparib (240). Olaparib is a well-established PARP-1 inhibitor in clinical development. Recently, CETSA was used to assess the binding of these two PARP-1 inhibitors and showed that iniparib failed to induce a thermal shift while binding of olaparib induced a large thermal shift of PARP-1 (240). Apparently, the mechanism of action of iniparib is not via physical binding to PARP-1; instead, iniparib may kill cancer cells by nonspecific effects.

1.8 Thesis overview

1.8.1 Rationale

ALK, which encodes a tyrosine kinase member of the insulin receptor superfamily, was initially discovered and characterized as a potent oncogenic driver in ALK-positive anaplastic large-cell lymphoma (30, 60). More recent studies have identified various genetic aberrations involving *ALK* in several types of human cancer, including neuroblastoma (NB), IMT and non-small cell lung cancer (16). Deregulation of ALK can be attributed to different forms of genetic alterations including chromosomal translocations, gene amplification, and activating mutations, all of which are believed to result in the constitutive activation of the ALK tyrosine kinase. In keeping with the pathogenetic importance of ALK in human cancer, inhibition of ALK using various pharmacologic agents have shown to be therapeutically effective in a subset of patients carrying ALK^+ tumors (16).

Crizotinib, the first ALK inhibitor used in the clinic, is known for its selective binding to ALK and has demonstrated remarkable efficacy against ALK^+ tumors occurring in mouse models as well as humans (40, 185). For example, crizotinib has shown striking anti-tumor activity in relapsed ALK^+ALCL patients (187, 188). However, a number of recent studies have demonstrated that the therapeutic benefits of crizotinib are variable among different types of ALK^+ cancer (24, 56, 189). The mechanisms underlying the differential clinical responses to crizotinib are not well understood. It is widely accepted that mutations within the *ALK* kinase domain can drive acquired resistance to crizotinib (16). On the other hand, while it was initially reported that the differential crizotinib sensitivity in EML4-ALK-expressing cells (in NSCLC) is related to the existence of the four EML4-ALK fusion variants (24), results from subsequent studies did not confirm the relationship between these EML4-ALK variants and crizotinib responses (192, 195). In addition, a wide range of crizotinib responsiveness was found in a cohort of ALK^+ NB patients in a phase 1 clinical trial (187). Subsequent studies have shown that crizotinib demonstrates a marginal benefit to a subset of ALK^+ NB patients (186, 196). Taken together, resistance to crizotinib remains to be a significant challenge in the clinic, and the mechanisms underlying this specific drug resistance is incompletely understood. In brief here, I hypothesize that ***the physical binding between***

crizotinib and ALK/ALK-variants is the determining factor of crizotinib sensitivity, and thus, the extent of crizotinib—ALK binding can be used to predict the biological responsiveness to crizotinib.

The paradigm of conventional cancer treatments hinged on the eradication of a presumably, biologically uniform cancer cell population. Recent studies have highlighted the importance of cancer stem cells (CSCs) as a major contributing factor to treatment failure, including primary drug resistance and relapses, seen in cancer patients (257). Generally, CSCs, whose existence has been demonstrated in various types of cancer, represent a small fraction of cells in the tumor bulk that drive tumorigenesis (238). In neuroblastoma, it has been recently reported that CSC-like cells can be identified based on their expression of a number of markers, such as CD133 and nestin (237). The discovery of the crucial role of different *ALK* aberrations as the driving oncogene in the pathogenesis of neuroblastoma has positioned ALK as the most promising tractable oncogene for targeted therapy in this disease (53, 54, 258). While acquired resistance to crizotinib (which is caused by post-treatment changes such as alteration in drug targets and activation of compensatory survival signaling pathways) is under extensive investigations (16), intrinsic resistance (which includes the factors that exist before treatment, such as the presence of cancer stem cells) is almost lacking in ALK^+ cancers. Here, I am going to investigate the crizotinib responsiveness among NB CSCs, as the correlation between the intrinsic crizotinib resistance and CSC-like cells in neuroblastoma has not yet been studied.

NB is the most common type of extra-cranial solid tumors in childhood, and it carries an overall poor clinical outcome despite intensive chemotherapy (150). Known prognostic factors that are associated with a worse clinical outcome include an age of >18 months, *MYCN* amplification, clinical stage, level of cellular differentiation, and DNA diploidy (259). ALK protein was found to be expressed in up to 60% of NB (166, 169), and it is believed that ALK is of pathogenetic importance in these tumors (51-54). There is mounting evidence that specific *ALK* mutations, with the ALK^{F1174L} mutation being the best characterized, are activating mutations and these mutated *ALK* are highly oncogenic (163). Nonetheless, these mutated *ALK* are found only in ~8% of NB patients (54). Meta-analysis studies also have identified the ALK^{F1174L} to be associated with *MYCN* gene amplification, a genetic marker of poor prognosis (166, 260). However, the significance of

ALK expression without specific alterations of the *ALK* gene, which occur in the majority of NB, is not as clear. Multiple reports have shown that ALK protein expression can be found equally in tumors with and without *MYCN* amplification (169). Overall, the role of ALK expression in NB needs to be characterized and defined.

1.8.2 Objectives

In chapter 2, my objective is to quantitatively measure the crizotinib—ALK binding, using the cellular thermal shift assay (CETSA), a recently described method that allows rapid and simple assessment of target engagement of drugs in a cellular context (240-242). If there is a correlation between crizotinib—ALK binding and crizotinib sensitivity in ALK-expressing cells, then mechanisms behind this will be investigated. Next, I asked two main questions. First, whether the differential crizotinib—ALK binding among different cell lines is due to the cell-type specific biochemical background and/or a difference in the efficiency of crizotinib transport into the cells. Second, whether ALK-binding proteins play a role in modulating crizotinib—ALK binding and crizotinib resistance.

In chapter 3, my objective is to assess the direct role of crizotinib—ALK binding based on the identification of a new level of intra-tumoral heterogeneity in an established experimental model using neuroblastoma. Specifically, I will investigate the existence of two phenotypically distinct cell subsets that differed in their responsiveness to a Sox2 reporter in neuroblastoma cell lines. Next, I will establish and validate the tumorigenic ability of these two cell subsets (i.e. reporter unresponsive (RU) cells and reporter responsive (RR) cells). Lastly, I will quantitatively measure the crizotinib—ALK binding in these two subsets.

In chapter 4, my objective is to achieve a better understanding of the role of ALK in neuroblastoma by examining the mRNA and protein expression of ALK in a cohort of NB. I am particularly interested in the expression of intron 19, since portions of this intron have been recently detected in unusual ALK transcripts in subsets of melanoma and in rare cases of lung cancer and IMT (49, 261-265). If this I19-containing ALK transcript is detected in NB, the biological and clinical significance of this *ALK* variant will be investigated.

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Chapter 2

The use of cellular thermal shift assay (CETSA) to study crizotinib resistance in ALK-expressing human cancers [1]

[1]A version of this chapter has been published as:

Alshareef A, Zhang HF, Huang YH, Wu C, Zhang JD., Wang P, El-Sehemy A, Fares M, Lai R. The use of cellular thermal shift assay (CETSA) to study crizotinib resistance in ALK-expressing human cancers. *Scientific Reports* 2016, 6, 33710. *Scientific Reports* Journal is an open-access journal, and copyright of the any paper published in this journal is retained by the author(s).

As first author of this paper, I prepared the first draft and revisions based on the suggestions and comments of the co-authors. I designed and performed most of the experiments described herein, except for the following: Zhang HF, Huang YH and Wu C performed a small portion of experiments, data shown in Figure 2.6, Figure 2.7C and Figure 2.8, and contributed in finalizing the manuscript. Zhang JD. and Wang P generated the two crizotinib-resistant ALK+ALCL cell lines. El-Sehemy A and Fares M prepared Figure 2.13 and wrote the computational analysis. Lai R provided numerous comments and final review of the manuscript before it was submitted for publication.

2.1 Introduction

Anaplastic lymphoma kinase (*ALK*), which encodes a tyrosine kinase member of the insulin receptor superfamily, was initially discovered and characterized as one of the two fusion gene partners identified in anaplastic large-cell lymphoma (ALCL) carrying the *t(2;5)* chromosomal abnormality (1). In ALCL, the catalytic domain of the ALK protein was fused with the amino terminus of nucleophosmin (NPM), and it was found that the NPM-ALK fusion protein results in constitutive activation of the ALK tyrosine kinase, thereby leading to deregulation of multiple cell signaling pathways and increased tumorigenicity (2). The signaling pathways of STAT3 and Wnt are among the best studied pathways in the context of NPM-ALK pathobiology, and both of these signaling pathways have been found to promote cell growth and survival of ALCL cells (3-7). Subsequent studies of ALCL and other types of human cancer have revealed various types of *ALK* gene aberrations and additional fusion partners of ALK (8, 9). For instance, the echinoderm microtubule-associated protein like 4 (*EML4*)-*ALK* fusion was identified in a small subset of non-small cell lung cancers (NSCLC) (10, 11). In neuroblastoma (NB), the most common and aggressive childhood malignancy, *ALK* has been found to be amplified or mutated at various locations (12-15). Specifically, gene amplification of *ALK* can be identified in 3.5% of NB tumors while activating mutations of *ALK* can be found in 8.4% of NB tumors in one study (12). The presence of any *ALK* aberrations in NB correlates with a short overall survival (16). In keeping with the pathogenetic importance of ALK, inhibition of ALK using pharmacologic agents or siRNA has been shown to result in cell cycle arrest and apoptosis in various forms of ALK-positive (ALK^+) human cancer (3).

Crizotinib is the first ALK inhibitor used in the clinic (17). Known for its selective binding to the inactive conformation of MET and ALK, crizotinib has demonstrated remarkable efficacy against ALK^+ tumors occurring in mouse models as well as humans (18). For instance, crizotinib has shown remarkable anti-tumor activity in relapsed ALK^+ALCL patients (19, 20). However, while crizotinib has been shown to be therapeutically efficacious in treating ALK^+ NSCLC patients, many of the treated patients showed disease progression within a year of therapy (21). A number of recent studies have demonstrated that the therapeutic benefits of crizotinib are variable among different types of ALK^+ cancer (16, 22, 23). The mechanisms underlying the differential clinical

responses to crizotinib are not well understood. Initial studies in small cohorts of patients have already shown that mutations within the *ALK* kinase domain can drive acquired resistance to crizotinib (8). In NSCLC, while it was initially reported that the differential crizotinib sensitivity in EML4-ALK-expressing cells is related to the existence of the four EML4-ALK fusion variants (23), results from subsequent studies did not confirm the relationship between these EML4-ALK variants and crizotinib responses (24, 25). In a phase 1 clinical trial, a wide range of crizotinib responsiveness was found in a cohort of ALK^+ neuroblastoma patients (19). Specifically, 34 patients were treated with crizotinib, and only 2 (6%) patients showed complete remission and 8 (23.5%) showed stable disease; the remaining 24 (70.5%) patients showed progressive disease despite intensive crizotinib treatment (19). A more recently published study also has shown that crizotinib demonstrates marginal benefit to a subset of ALK^+ neuroblastoma patients (26, 27). Taken together, resistance to crizotinib remains to be a significant challenge in the clinic, and the mechanisms underlying this specific drug resistance is incompletely understood.

In this study, we aimed to study the biology of crizotinib resistance, by correlating various structures/forms of ALK in a panel of ALK^+ cancer cell lines and the *in vitro* sensitivity to crizotinib. We hypothesize that the physical binding between crizotinib and ALK (and its variants) is the determining factor of crizotinib sensitivity, and thus, the extent of crizotinib—ALK binding can be used to predict the biological response to crizotinib. To quantitatively measure the crizotinib—ALK binding, we employed the cellular thermal shift assay (CETSA), a recently described method that allows rapid and simple assessment of target engagement of drugs in a cellular context (28-30). Our results have led us to conclude that the crizotinib—ALK binding measurable by CETSA is useful in predicting crizotinib sensitivity in ALK^+ cancer cells, and crizotinib—ALK binding is in turn dictated by structure of ALK and some of its binding partners.

2.2 Materials and Methods

2.2.1 Cell lines

The characteristics of the ALK^+ALCL cell lines (Karpas 299 and SupM2) have been previously described (31). The four ALK-positive neuroblastoma cell lines (NB1, IMR32, GOTO and SK-N-

SH) used in this study were kind gifts from Dr. Rosline Godbout (Department of Oncology, University of Alberta). The non-small cell lung cancer cell line, H2228, was a kind gift from Dr. Ming Tsao (Ontario Cancer Institute). The characteristics of the ALK^+ALCL cell lines (Karpas 299 and SupM2) have been previously described (31). The MCL cell line (SP53) has been previously described (32). All cell lines were maintained in RPMI 1640 (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Grand Island, NY, USA). All cell lines have been authenticated using short tandem repeat DNA profiling (from TCAG Genetic Analysis Facility, Toronto, CA).

2.2.2 Cellular thermal shift assay (CETSA)

The ability of compounds to interact with, and thereby stabilize the target in intact cells, was analyzed essentially as described by Molina et al (28). Briefly, cells cultured in 100 x 20 mm tissue culture dishes at 90% confluence were treated with media containing DMSO or crizotinib (doses used as described in the text) for 6 hours. For suspension cells, 1 x 10^6 cells needed per CETSA-temperature (4 x 10^6 needed for the 4 temperatures). After treatment, adherent cells were detached with trypsin, all cells collected by centrifugation and subsequently resuspended in PBS supplemented with protease inhibitor. The cell suspension was aliquoted into four PCR tubes and heated for 3 minutes to 50, 51, 52 or 53°C. Subsequently, cells were lysed using liquid nitrogen and two repeated cycles of freeze-thaw. Precipitated proteins were separated from the soluble fraction by centrifugation at 17,000g for 20 minutes. Soluble proteins, collected in the supernatant, were kept at −80°C until Western blot analysis. Equal amount of proteins were loaded onto 6% SDS–PAGE gels, transferred to nitrocellulose membranes and analyzed using the ALK-antibody from Cell Signaling at a concentration of 1:1000. The ALK protein was detectable by Western blots at relatively low temperatures (i.e. ≤51°C). When the temperature increased, the ALK protein started to aggregate and gradually disappeared from the supernatant after centrifugation. In the event of substantial crizotinib—ALK binding, ALK was relatively protected and it remained in the supernatant at a relatively high temperature (i.e. ≥52°C). Protein expression levels on Western blots were quantified by densitometry analyses using the ImageJ software. The densitometry values were determined after normalization to the ALK band at 50°C.

2.2.3 Reagents, Plasmids and siRNA transfection

Crizotinib (PF-2341066), was purchased from Sigma-Aldrich (Oakville, Ontario, Canada). The concentration of the DMSO stock solution of crizotinib was 1mM. The HSP90 inhibitor, NVP-AUY922, was purchased from Selleck. Each compound was dissolved in DMSO for cell culture experiments. The pcDNA3-FLAG-ALK wild-type and mutated (F1174L mutation) were kindly provided by Dr. Junko Takita (The University of Tokyo, Tokyo, Japan). For β-catenin knockdown, β-catenin specific ON-Target Plus SMARTpool small interfering RNA (siRNA) and scramble control were purchased from Thermo Scientific (Chicago, USA). A final concentration of 20 nM siRNA was used in all β-catenin knockdown experiments.

2.2.4 Cell viability analysis

Cell viability was performed as previously described (33, 34). Briefly, cells were plated in 96-well plates, 2000 cells/well. 24h after plating, cells were cultured in medium containing different doses of crizotinib (i.e. 0nM, 250nM, 500nM, 750nM, 1000nM, 1500nM and 2000nM). Cell viability was measured using the CellTiter 96AQueous One Solution Cell Proliferation Assay (Promega), also known as MTS assay, 72 hours after drug treatment according to the manufacturer's protocol. Absorbance at 490nm was measured using a microplate reader (BMG Labtech, Ortenberg, Germany). The inhibitory concentration at 50% (IC_{50}) was calculated by Graphpad Prism (La Jolla, CA). For the β-catenin knockdown experiment, IMR32 and SK-N-SH cells were transfected with either scrambled siRNA or β-catenin siRNA, followed by treatment with the above-mentioned doses of crizotinib for 72 hours.

2.2.5 Western blot and Co-Immunoprecipitation analysis

Western blot assay was performed using standard procedure. Briefly, the cells were washed with cold 1 X PBS and lysed in 1X RIPA lysis buffer (ThermoFisher Scientific) containing protease inhibitor cocktail (Sigma Aldrich) and phosphatase inhibitor cocktail (Sigma Aldrich). After incubation on ice for 30 minutes, the cell lysates were subjected to centrifuge at 17,000 g for 20 minutes at 4°C, and the supernatants were collected. The protein concentration was assessed using

BCA protein assay kit (Bio-Rad, Hercules, CA, USA). The protein lysates were then denatured by heating with 4 X loading buffer (240 mM Tris-HCL pH 6.8, 40% glycerol, 8% SDS, 0.04% bromophenol blue, 5% β-mercaptoethanol) at 100°C for 5 minutes. Six-percent or 10% SDS-polyacrylamide gel (SDS-PAGE) was used. After electrophoresis and transfer to nitrocellulose membranes (Bio-Rad) by electroblotting, the membranes were probed with primary antibodies (1:1000, overnight at 4°C) and secondary antibodies (1:2000, one hour at room temperature), followed by the enhanced chemiluminescene detection system (Bio-Rad).

Co-immunoprecipitation was performed and described briefly as below. Cells were harvested and washed twice with cold 1X PBS, then were lysated with CelLytic M buffer (Sigma Aldrich) containing protease inhibitor cocktail (Sigma Aldrich) and phosphatase inhibitor cocktail (Sigma Aldrich). After incubation on ice for 30 minutes, the cell lysates were proceeded to centrifuge at 17,000g for 15 minutes at 4°C. The supernatants (protein lysates) were collected and quantitated by using protein assay kit (Bio-Rad). Then the protein lysates were incubated with appropriate amount of primary antibody and rotated at 4 °C for overnight. Next day, the 1X PBS-washed protein A Plus / Protein G agarose (ThermoFisher) were added to the protein lysates and rotated at 4°C for additional 3 hours. Lastly, the protein lysates were centrifuged at 8000g for 3 minutes and the agaroses were kept and washed gently with cold 1 X PBS for 3 times. Then the agaroses were resuspended in 50 µl 4X loading buffer and heated for 5 minutes at 100°C, followed by centrifuge at 17,000g for 15 minutes. The supernatant were collected and thereafter were proceed to SDS-PAGE. Antibodies against for phosphorylated ALKY1604 (#3341), ALK (C26G7), phosphorylated STAT3Y705 (D3A7) and β-catenin Antibody (#9562) were purchased from Cell Signaling Technology (Danvers, MA). Antibodies against Vinculin (N-19) and STAT3 (H-190) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2.6 Broad-Novartis Cancer Cell Line Encyclopedia

The Broad Institute and the Novartis Institutes for Biomedical Research have collaborated to generate gene expression data on about 1000 cancer cell lines in a project termed the *Cancer Cell Line Encyclopedia* (CCLE) (35). Gene expression data for CTNNB1 (β-catenin) was extracted from CCLE_Expression_Entrez_2012-10-18.res. We also extracted crizotinib responsiveness for

seven out of the nine analyzed ALK-expressing cell lines published in CCLE database; including IMR32, SK-N-BE(2), SK-N-DZ, KP-N-SI9s, Kelly, SK-N-SH, Karpas 299, SupM2 and H2228 (35). Through the CCLE Terms of Access, we declare that, "those who carried out the original analysis and collection of the data bear no responsibility for the further analysis or interpretation of it."

2.2.7 Molecular modeling and docking studies

PDB crystal structure files of ALK, crizotinib [PDB ID: 2XP2] (36), β-catenin [PDB ID: 1LUJ] (37) and HSP90 [PDB ID: 1US7] (38) were downloaded from the public Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB) online database (www.rcsb.org/pdb) (39). Protein structure files were handled using Pymol (40) and ICM-browser Molsoft (41, 42) molecular editing programs in order to visualize the structures and prepare them for docking according to standard procedures; for every file the ligand was removed freeing the corresponding proteins of interest, then missing hydrogen atoms were added to the structure. For docking studies, we used Cluspro protein-protein online docking server provided by Boston University (http://cluspro.bu.edu/) according to the standard protocol and recommended specifications, and using cluster sizes to rank predicted binding models (43-46).

2.2.8 Reverse transcriptase-polymerase chain reaction (RT-PCR) and sequencing for secondary mutation

Total RNA extraction was performed with the Qiagen RNeasy Kit (Qiagen Canada) according to the manufacturer's protocol. Briefly, 1 µg of RNA was reverse transcribed using Superscript II (Life Technologies) according to the manufacturer's protocol. One µL of the resulting cDNA mixture was added to the Platinum SYBR Green qPCR (Life Technologies) and amplified with ALK specific primers. The ALK primer sequences are as follows: forward) 5'-CTTTGGGGAGGTGTATGAAGG-3' and reverse) 5'-GGCACTTTCTCTTCCTCTTCC-3'. The expected PCR product size is 858 bp. Sequencing was performed at The Applied Genomics Centre (TAGC), University of Alberta.

2.2.9 Statistical analysis

All the statistical analyses were performed using the GraphPad Prism 5.1 program. Student *t* test was used to calculate *p* values. Results are presented as mean ± standard deviation of 3 biological replicates (assays performed on 3 different days). The Fisher's exact test was used to correlate crizotinib sensitivity with crizotinib—ALK binding among the 7 ALK^+ cancer cell lines. The nonparametric Spearman's rank correlation coefficient was applied to evaluate the correlation between crizotinib IC_{50} values and β-catenin mRNA levels.

2.3 Results

2.3.1 Crizotinib—ALK binding correlates with crizotinib sensitivity in ALK-expressing cells

First, we asked if there is a correlation between crizotinib—ALK binding and crizotinib sensitivity in ALK-expressing cells. To answer this question, we performed CETSA using 7 ALK-expressing cell lines, including 2 ALK-positive anaplastic large cell lymphoma (ALK^+ALCL) cell lines (Karpas 299 and SupM2), 4 neuroblastoma cell lines (NB1, IMR32, GOTO and SK-N-SH) and one non-small cell lung cancer cell line (H2228), and correlated these results with the crizotinib sensitivity (i.e. inhibitory concentration at 50%, IC_{50}). The expression of the ALK proteins and their phosphorylation status in these 7 cell lines are illustrated in **Figure 2.1**. In the left panel in which the results from the 4 neuroblastoma cells lines are illustrated, we found the 220 kDa band, which represents the full-length ALK protein, and/or several bands at lower molecular weight (e.g. 140 kDa). These findings are in accordance with the published findings from other groups (47). Western blots using anti-pALK showed essentially a similar pattern as that of anti-ALK. In the right panel where the results of the three cell lines carrying ALK fusion proteins are shown, we found the ALK fusion proteins at their expected molecular weights. Specifically, NPM-ALK present in SupM2 and Karpas 299 was located at approximately 80 kDa, whereas EML4-ALK

present in H2228 was found at approximately 89 kDa, as reported previously (48). SP53, a mantle cell lymphoma cell line, served as the negative control for ALK and pALK.

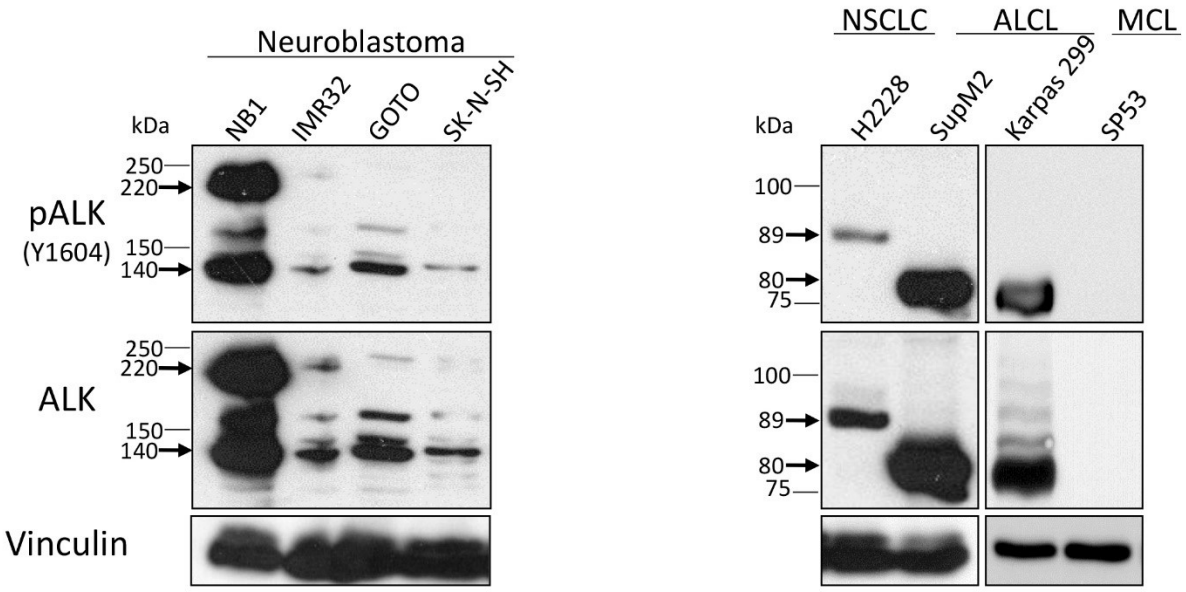

Figure 2.1 Expression levels of pALK and ALK in 7 ALK-expressing cancer cell lines.

The four ALK-positive neuroblastoma cell lines (NB1, IMR32, GOTO and SK-N-SH) express full-length ALK (220 kDa) and/or several bands at lower molecular weight (e.g. 140 kDa). The non-small cell lung cancer (NSCLC) cell line, H2228, expresses EML4-ALK translocation variant 3 (89 kDa). The two ALK^+ALCL cell lines (SupM2 and Karpas 299) express NPM-ALK (80 kDa). SP53, a mantle cell lymphoma (MCL) cell line, was used as a negative control and showed no ALK expression.

CETSA results and the IC_{50} data (derived from the literature as well as our own studies) are summarized in **Table 2.1**. For the purpose of this study, cell lines with an IC_{50} of ≤56 nM, including the two ALK^+ALCL cell lines and NB1, were considered crizotinib-sensitive; the other 4 cell lines that carried an IC_{50} of >56 nM were considered crizotinib-resistant. Results from CETSA are illustrated in **Figure 2.2A and 2.2B**. As detailed in Materials and Methods, crizotinib—ALK binding was assessed 'positive' if there was significantly more ALK in the crizotinib-treated group compared to the DMSO-treated group at 52°C detectable by Western blots. In comparison, crizotinib—ALK binding was assessed 'negative' if there was no significant difference in the ALK expression level between the two groups at 52°C. Statistical analysis using Fisher exact test has revealed that the correlation between crizotinib sensitivity and crizotinib—ALK binding among these 7 cell lines is significant (P=0.029). Overall, these results suggest that a lack of crizotinib—ALK binding is a major contributing factor to crizotinib resistance in ALK-expressing cancer cells.

Table 2.1 Summary of the IC_{50} data (derived from the literature as well as our own studies) and the CETSA results.

Cancer type	Cell line	ALK form	IC_{50} in nM (Standard Deviation)	Reported IC_{50} in nM (reference)	Crizotinib sensitivity	CETSA at 52°C
ALCL	SupM2	*NPM-ALK*	40 (± 9)	56 (49)	Sensitive	Positive binding
	Karpas 299	*NPM-ALK*	45 (± 7)	32 (50)	Sensitive	Positive binding
Neuroblastoma	NB1	Full-length $ALK^{Amplified}$	10 (± 4)	10 (51)	Sensitive	Positive binding
	IMR32	Full-length $ALK^{wild\text{-}type}$	970 (± 134)	740 (52)	Resistant	Negative binding
	GOTO	Full-length $ALK^{wild\text{-}type}$	1817 (± 200)	> 5000 (35)	Resistant	Negative binding
	SK-N-SH	Full-length ALK^{F1174L}	631 (± 83)	1900 (35)	Resistant	Negative binding
NSCLC	H2228	*EML4-ALK variant 3*	984 (± 96)	871 (53) & 834 (52)	Resistant	Negative binding

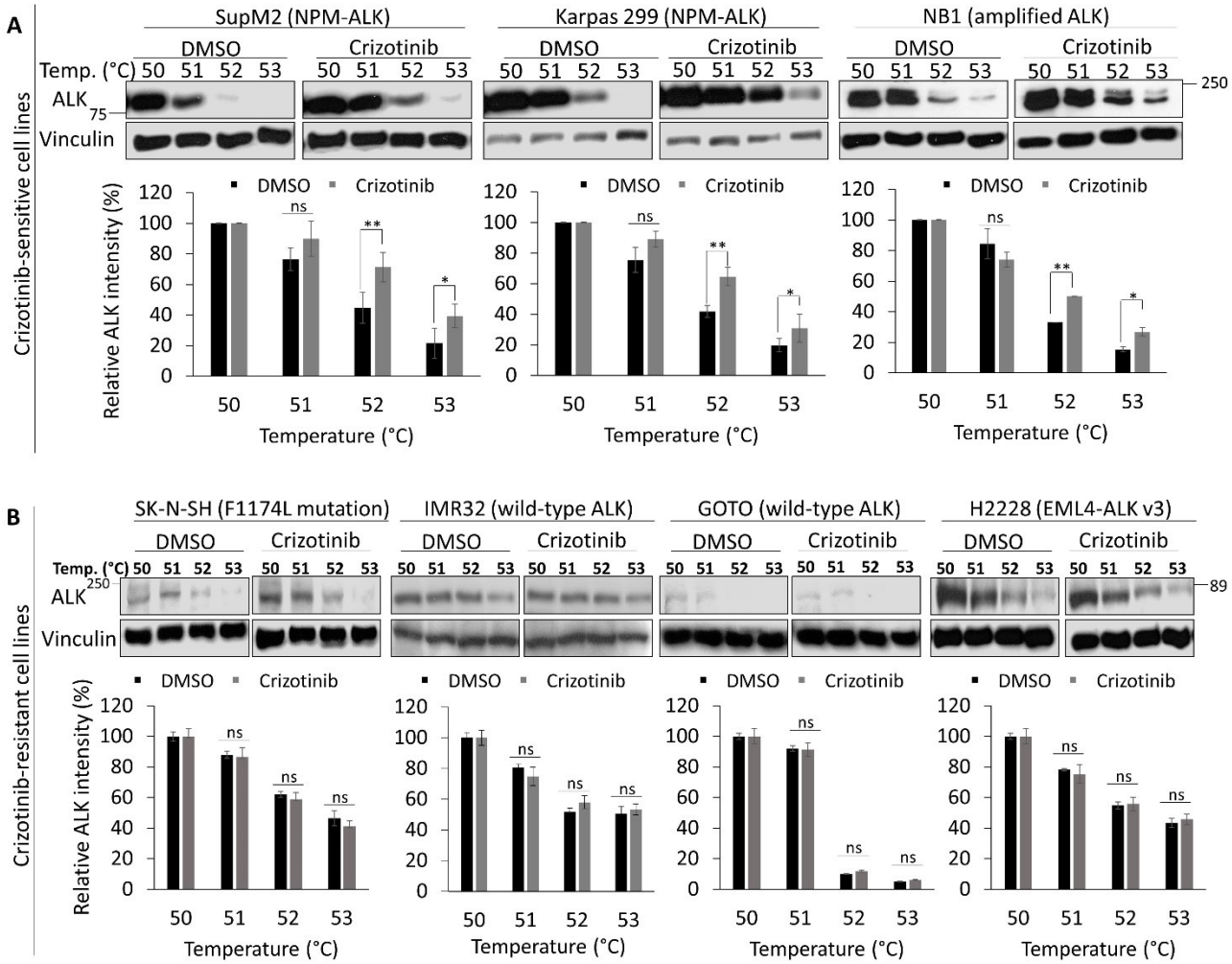

Figure 2.2 Crizotinib, an ALK inhibitor, binds to ALK in crizotinib-sensitive cell lines but not in crizotinib-resistant cell lines.

CETSA was performed to measure the binding ability of crizotinib to different ALK forms in crizotinib-sensitive and crizotinib-resistant cell lines. **A.** Three crizotinib-sensitive cell lines, include SupM2 (n=5), Karpas 299 (n=3) and NB1 (n=3), were treated with 50 nM crizotinib for 6 hours. Representative ALK Western blots of each cell line are shown on the upper panel. **B.** Four crizotinib-resistant cells, include SK-N-SH (n=3), IMR32 (n=3), GOTO (n=3) and H2228 (n=3), were treated with 2000 nM crizotinib for 6 hours. Representative ALK Western blots of each cell line are shown on the upper panel. Vinculin level was blotted as a loading control. Data are presented as mean ± SD. **P*<0.05, ***P*<0.01, Student's *t* test.

2.3.2 Differential crizotinib—ALK binding is dictated by the ALK structure

We then asked if increasing the concentration of crizotinib will promote crizotinib—ALK binding in crizotinib-resistant cell lines. As shown in **Figure 2.3**, increasing the concentrations of crizotinib in two crizotinib-sensitive cell lines (SupM2 and NB1) resulted in an appreciable increase in the stabilization of ALK at 52°C. In contrast, increasing the concentrations of crizotinib in three crizotinib-resistant cell lines (SK-N-SH, IMR32 and H2228) consistently failed to yield any detectable change to the ALK stabilization at 52°C. Correlating with these findings, we noted that the viability of both sensitive cell lines dropped by an average of 65% when the crizotinib concentration at their IC_{50}'s were doubled. In contrast, the viability of the three resistant cell lines dropped by an average of only 30% when the crizotinib concentrations at their IC_{50}'s were doubled. Taken together, these findings further support the concept that a lack of crizotinib—ALK binding is a major contributing factor to crizotinib resistance in ALK-expressing cancer cells.

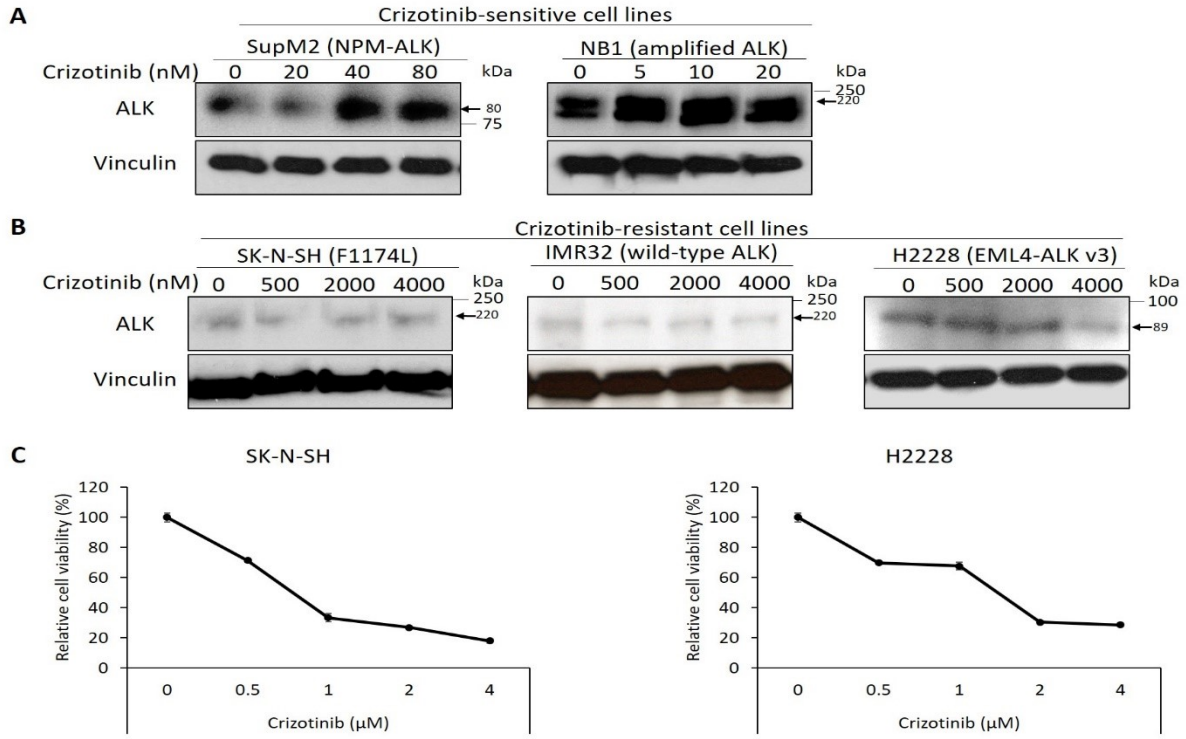

Figure 2.3 Crizotinib binds to ALK in crizotinib-sensitive cell lines while no binding is observed in crizotinib-resistant cell lines.

A. Two crizotinib-sensitive cell lines (SupM2 and NB1) demonstrated a dose-dependent increase of stabilized ALK using CETSA assay at 52°C upon crizotinib treatment. **B**. Three crizotinib-resistant cell lines (SK-N-SH, IMR32 and H2228) showed no ALK stabilization when treated with crizotinib at double IC_{50} dosages. **C.** Cell growth was measured using MTS assay (n=3).

To assess whether the differential crizotinib—ALK binding among different cell lines is due to the cell-type specific biochemical background and/or a difference in the efficiency of crizotinib transport into the cells, we transiently transfected *NPM-ALK* into 3 crizotinib-resistant cell lines (SK-N-SH, IMR32 and H2228). By CETSA, we found substantial crizotinib-NPM-ALK binding (**Figure 2.4**). In the same experiments, there was no substantial binding between crizotinib and the native forms of ALK. These results support the concept that the differential crizotinib sensitivity and crizotinib—ALK binding is likely specific to the structure/biochemistry of ALK structure, but not related to a lack of crizotinib transport into the cells or cell-type specific biochemical environment.

To further substantiate these findings, we performed additional experiments in which 3 different forms of ALK (NPM-ALK, full-length wild-type ALK and full-length, mutated ALK^{F1174L}) were transfected in GP293 cells (**Figure 2.5**). As shown in **Figure 2.6**, only NPM-ALK was bound by crizotinib and its pALK signals were largely abrogated with crizotinib treatment. In contrast, the same treatment in cells transfected with the full-length wild-type *ALK* or ALK^{F1174L} did not result in any substantial change to the ALK stabilization and only a partial decrease in pALK. Thus, these results also support the concept that crizotinib—ALK binding is primarily dependent on the ALK structure.

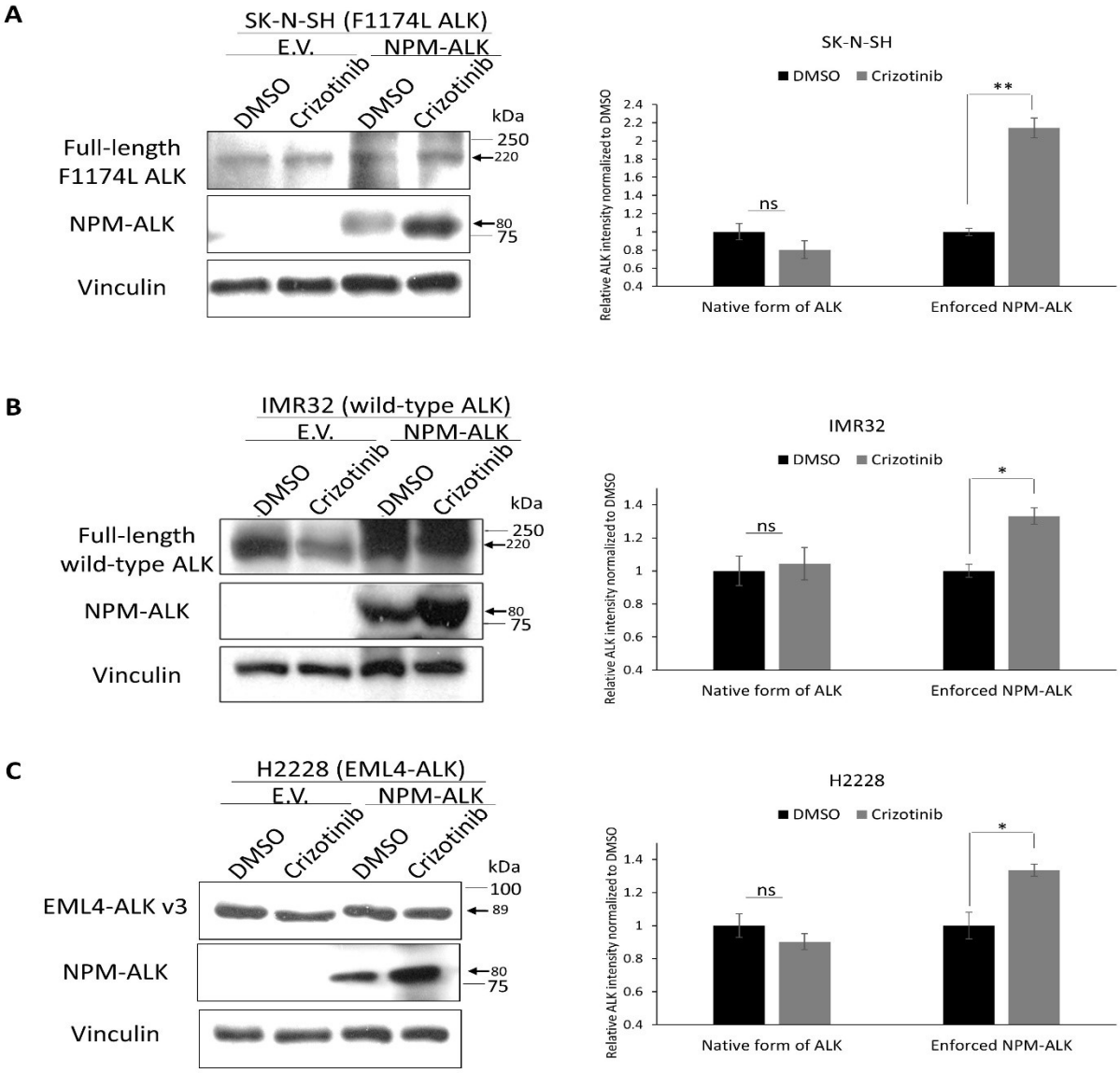

Figure 2.4 Differential sensitivity to crizotinib, through physical binding, is specific to ALK structure.

(A-C) Enforced expression of NPM-ALK into crizotinib-resistant cell lines (i.e. SK-N-SH, IMR32, and H2228) showed stabilization of NPM-ALK but not the native ALK. CETSA assay was performed at 52°C. Representative Western blots are shown on the left side and the densitometry quantification data from 3 independent experiments are shown on the right side. Data are presented as mean ± SD. **P*<0.05, ***P*<0.01, Student's *t* test.

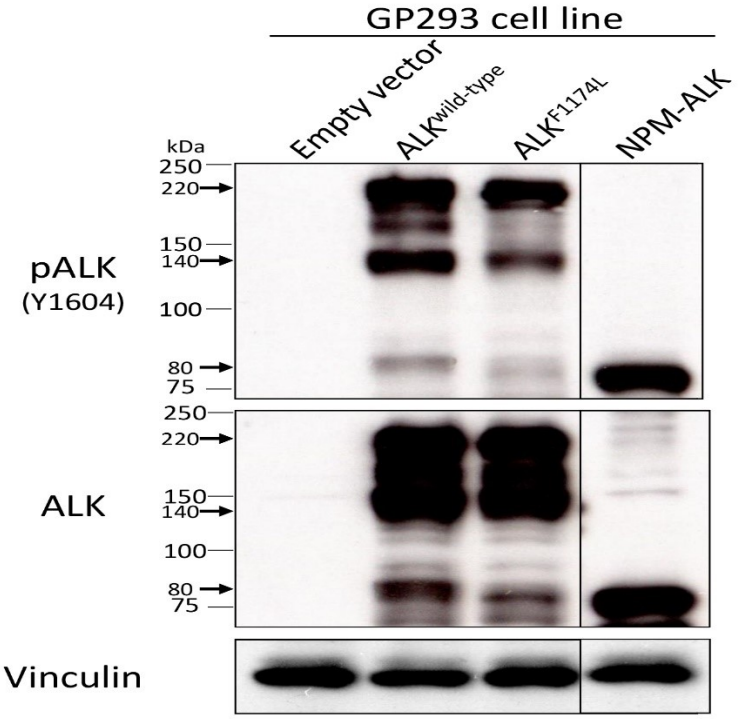

Figure 2.5 Enforced expression of various forms of ALK-expression plasmids (full-length wild-type ALK, full-length F1174L mutated ALK and wild-type NPM-ALK) in GP293 cells was determined by Western blots.

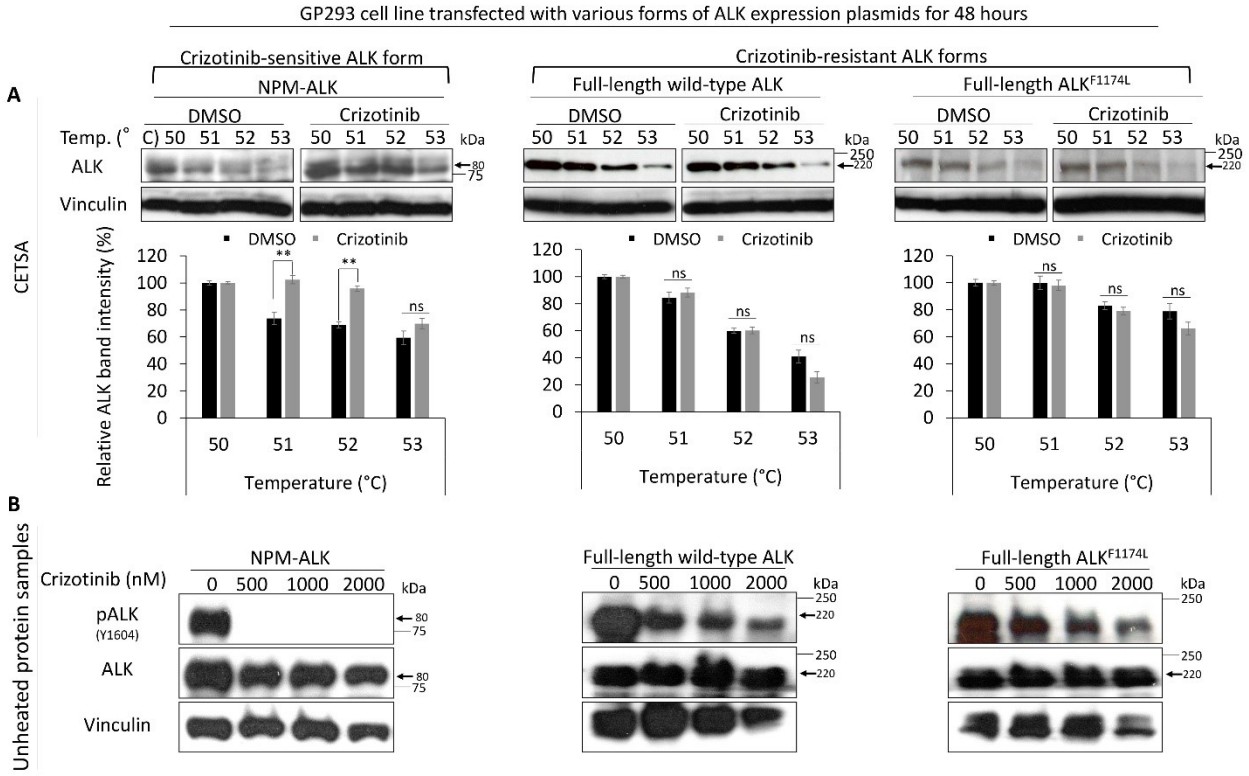

Figure 2.6 The ALK structure is the main determinant of crizotinib responsiveness and is correlated with inhibition of ALK phosphorylation upon enforced expression in the GP293 cell model.

A. The CETSA assay results showed crizotinib stabilized NPM-ALK at 51°C and 52°C while unable to stabilize full-length wild-type ALK or full-length mutated ALK (F1174L) at the same temperatures. **B.** Crizotinib treatment completely abrogated ALK phosphorylation in NPM-ALK but not the other two forms of ALK, i.e. full-length wild-type ALK and full-length F1174L ALK. Experiments were performed in triplicate. Data are presented as mean ± SD. $^{**}P<0.01$, Student's *t* test.

2.3.3 Abrogation of crizotinib—ALK binding in crizotinib-resistant ALK$^+$ALCL cell lines

To reinforce the concept that the crizotinib—ALK binding pattern revealed by CETSA is useful in predicting crizotinib sensitivity, we established two crizotinib-resistant ALK$^+$ALCL cell clones derived from Karpas 299 and SupM2. These cell clones were established by subjected them to increasing concentrations of crizotinib over a few weeks, reaching a final concentration of 500 nM for both cell lines.

To understand the mechanism of resistance in these crizotinib-resistant cell clones, we sequenced the *NPM-ALK* mRNA expressed in these cells; specifically, the segment between exon 20 to exon 29 from the *ALK* domain, which includes the kinase domain, was examined. As shown in **Figure 2.7**, four secondary mutations were identified in crizotinib-resistant ALK$^+$ALCL cell lines, with one mutation (G329A) occurring in Karpas 299 cells while 3 mutations (G262R, K551R and D589E) were observed in SupM2. The mutations at G329 in the *NPM-ALK* fusion (equivalent to G1269 in the full-length ALK) and G262 in the *NPM-ALK* fusion (equivalent to G1202 of the full-length ALK) are located in the tyrosine kinase domain. In accordance with our hypothesis, crizotinib—ALK binding was detectable only in crizotinib-sensitive, parental cell clones but not crizotinib-resistant cell clones (**Figure 2.7**). All of these acquired crizotinib-resistant cell lines showed a substantially lesser degree of pALK inhibition upon crizotinib treatment (**Figure 2.7**). To our knowledge, this is the first experimental evidence supporting the concept that secondary mutations of ALK contribute to crizotinib resistance by abrogating crizotinib—ALK binding. These results further support that the ALK structure (e.g. mutations) dictates crizotinib—ALK binding and crizotinib sensitivity.

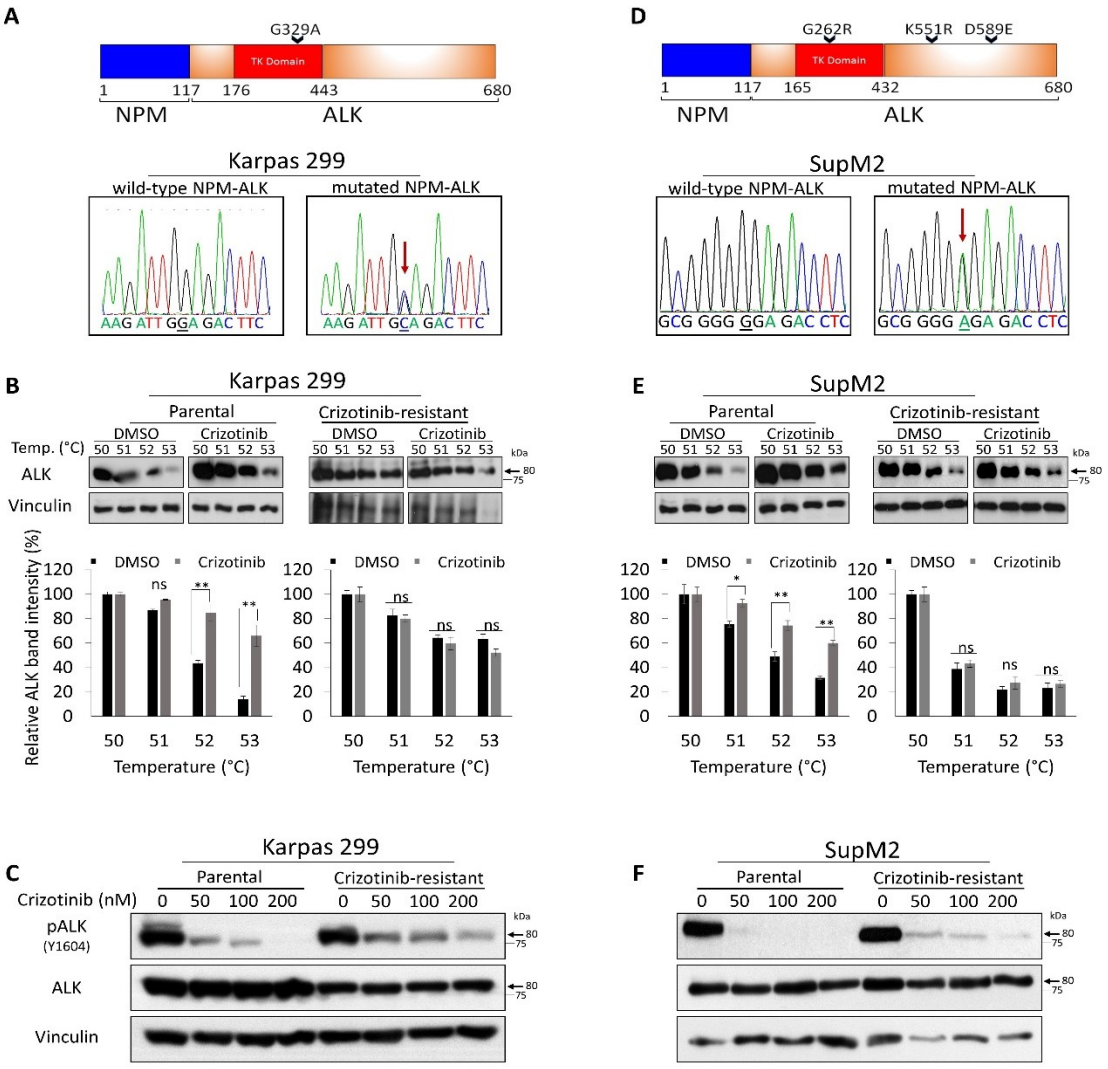

Figure 2.7 Identification of NPM-ALK secondary mutations in crizotinib-resistant ALK$^+$ALCL cell lines that abrogate the binding between crizotinib and ALK.

A. Sequencing of ALK kinase domain coding fragment in crizotinib-sensitive and crizotinib-resistant ALCL cell lines. Schematic of ALK kinase domain mutations associated with acquired resistance to crizotinib. **A** and **D** show the electropherograms of NPM-ALK cDNA from parental Karpas 299, crizotinib-resistant Karpas 299, parental SupM2, and crizotinib-resistant SupM2 cells. **B** and **E** show that crizotinib—ALK binding can be detected using CETSA assay in crizotinib-sensitive cells but not in crizotinib-resistant clones. **C** and **F** show that crizotinib treatment could substantially inhibit pALK in crizotinib-sensitive cells, while only partial pALK inhibition was observed in crizotinib-resistant clones. Data are presented as mean ± SD. N=3, *P<0.05, **P<0.01, Student's t test.

2.3.4 The role of β-catenin in modulating crizotinib—ALK binding and crizotinib-resistance

It has been published that ALK has a large number of binding proteins (6, 7). We hypothesized that some of these binding proteins might play a role in modulating crizotinib—ALK binding and crizotinib resistance. To this end, we compared the expression/binding levels of various known ALK-binding proteins between crizotinib-resistant and crizotinib-sensitive cell lines. As shown in **Figure 2.8A**, we found that β-catenin was expressed higher in all 4 resistant cell lines (IMR32, GOTO, SK-N-SH and H2228) as compared to two sensitive cell lines (SupM2 and NB1). As shown in **Figure 2.8B,** by immunoprecipitation, the ALK—β-catenin interaction was detectable in all cell lines examined, although the level of β-catenin pulled down with ALK was substantially higher in the three crizotinib—resistant cell lines when compared to crizotinib-sensitive cell lines. This difference is highlighted when we compared GOTO (which expressed a relatively low level of ALK but a high level of β-catenin pull-down) with NB1 or SupM2 (relatively high level of ALK but a low level of β-catenin pull-down).

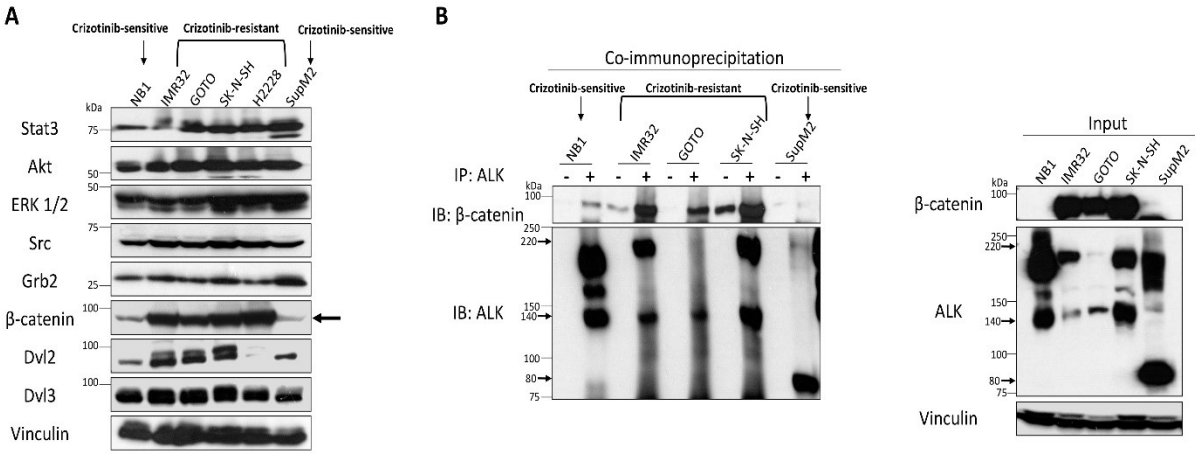

Figure 2.8 β-catenin interacts with ALK and shows of higher expression levels in crizotinib-resistant cell lines.

A. Screening of a panel of known ALK-effector proteins identified β-catenin as a protein that was highly expressed in crizotinib-resistant cell lines (i.e. IMR32, GOTO, SK-N-SH, and H2228). Please note that the Western blot from figure 1 was analyzed here against indicated antibodies. **B.** Left panel, ALK pull-down experiment, without ALK antibody (-) or with ALK antibody (+), showed substantial ALK-β-catenin binding only in crizotinib-resistant cell lines (i.e. IMR32, GOTO and SK-N-SH). Right panel, the input for the co-immunoprecipitation.

In keeping with the concept that β-catenin is important in regulating crizotinib—ALK interaction and crizotinib resistance, we subjected two crizotinib-resistant cell lines (IMR32 and SK-N-SH) to β-catenin siRNA knockdown for 72 hours, and we performed CETSA assay. As shown in **Figure 2.9**, crizotinib stabilized ALK upon β-catenin siRNA knockdown as compared to the negative controls. Importantly, restoration of crizotinib—ALK binding induced by β-catenin knockdown significantly sensitized IMR32 and SK-N-SH to crizotinib, lowering the IC_{50} from 1220 nM (scrambled siRNA) to 467 nM (i.e. a 62% decrease) and from 764 nM to 336 nM (i.e. a 57% decrease), respectively.

Of note, β-catenin siRNA knockdown alone (for 72 hours) did not significantly affect the cell growth of both cell lines (**Figure 2.10**). To substantiate these finding, we repeated the same experiment using our generated crizotinib-resistant SupM2 cell clone. As shown in **Figure 2.9C**, these cells were sensitized to crizotinib upon β-catenin siRNA knockdown, with the IC_{50} lowered from 1182 nM (scrambled siRNA) to 456 nM (i.e. a 62% decrease).

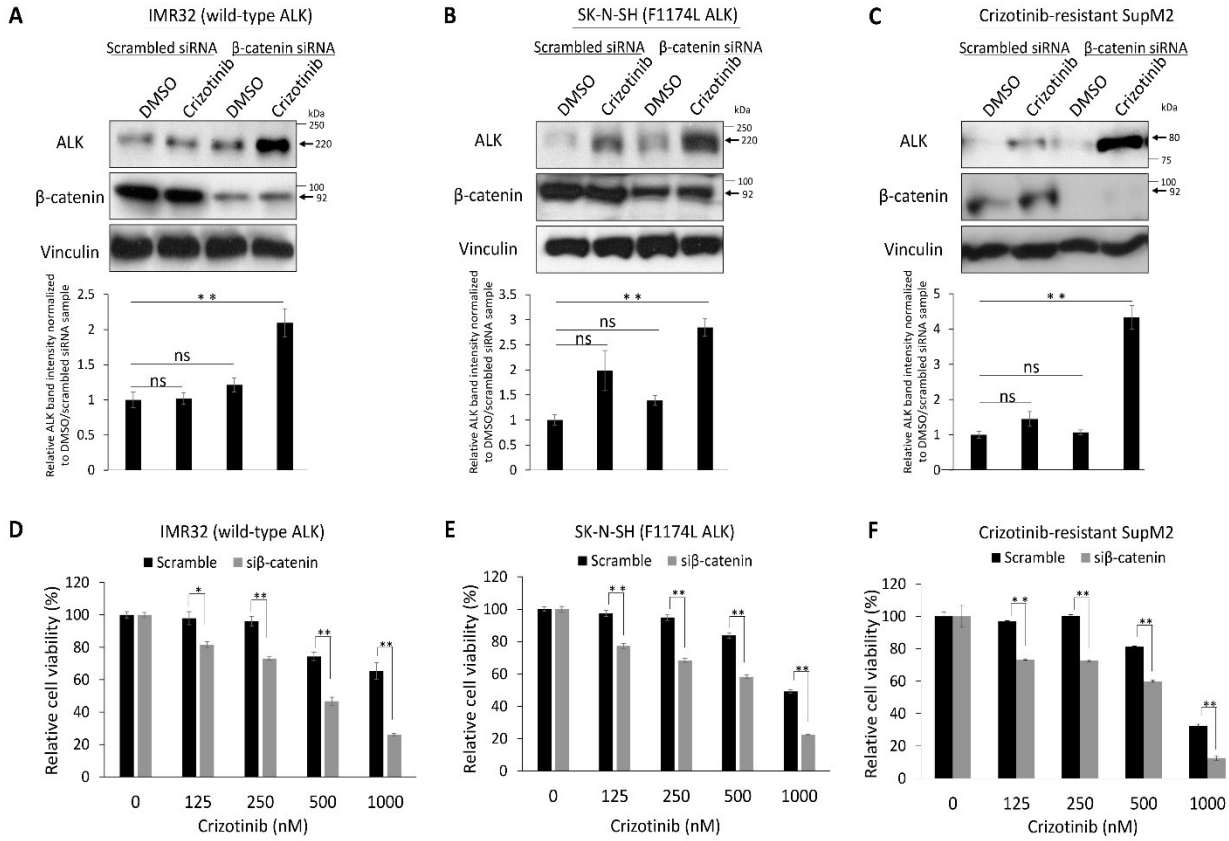

Figure 2.9 β-catenin siRNA knockdown restores crizotinib—ALK binding and significantly sensitizes crizotinib-resistant cell lines to crizotinib treatment.

(A-C) show that β-catenin siRNA knockdown significantly restored crizotinib—ALK binding upon crizotinib treatment in comparison to scrambled siRNA treatment. CETSA was performed at 52°C. **(D-F)** show that β-catenin siRNA knockdown significantly sensitized crizotinib-resistant cells to crizotinib treatment in comparison to scrambled siRNA treated cells. Data are presented as mean ± SD. N=3, **P*<0.05, ***P*<0.01, Student's *t* test.

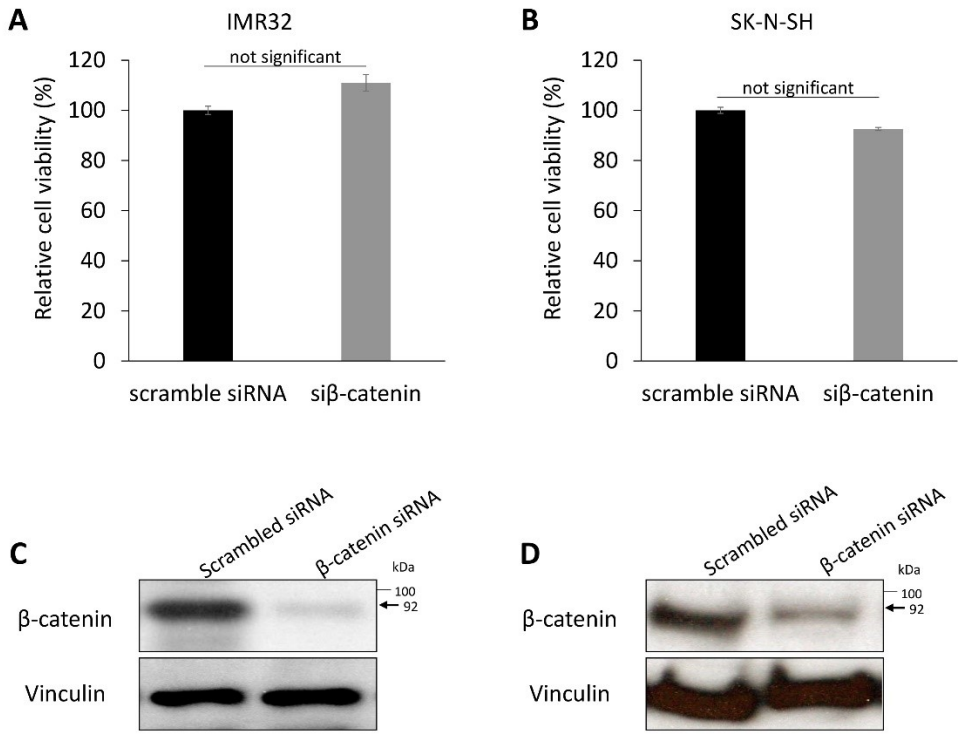

Figure 2.10 β-catenin knockdown does not affect the cell viability of IMR32 and SK-N-SH after 72 hours.

A and **B** show the minimal effect of β-catenin siRNA knockdown on the cell viability of the two crizotinib-resistant cell lines (IMR32 and SK-N-SH). **C** and **D** show representative Western blots after β-catenin knockdown in IMR32 and SK-N-SH cells.

To prove the specificity of β-catenin, we examined another known ALK-binding protein, namely HSP90, which is a chaperone reported to play an important role in protein folding (54). The choice of using HSP90 is also related to the fact that HSP90 inhibitor has been shown to be highly effective against ALK^+ lung cancer cells as well as ALK^+ALCL cells in preclinical and clinical studies (55, 56). As shown in **Figure 2.11**, treatment of two crizotinib-resistant cell lines (SK-N-SH and H2228) with increasing doses of an HSP90 inhibitor (NVP-AUY922) did not substantially alter the crizotinib—ALK binding or the crizotinib susceptibility in the crizotinib-resistant cell lines, lowering the IC_{50} from 752 nM (DMSO) to 670 nM (i.e. an 11% decrease) in SK-N-SH and from 974 nM to 836 nM (i.e. a 15% decrease) in H2228.

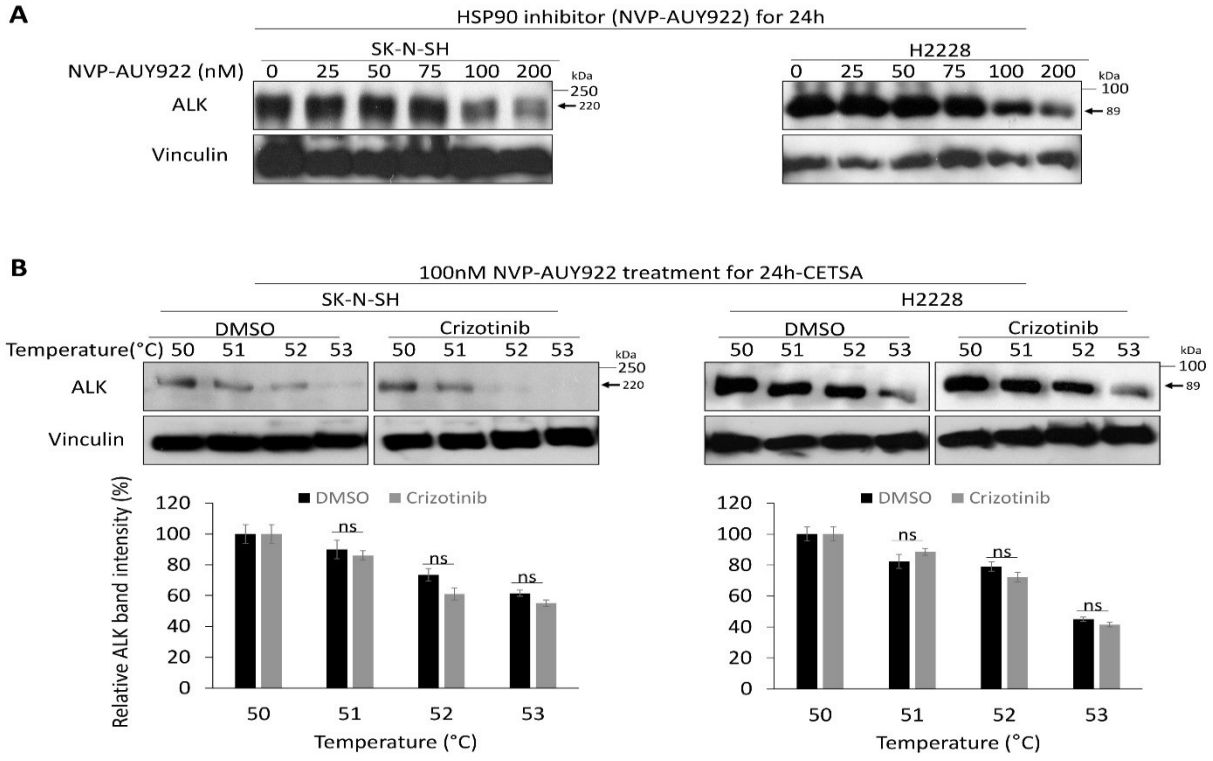

Figure 2.11 HSP90 inhibition does not facilitate the crizotinib—ALK binding in crizotinib-resistant cell lines.

A. NVP-AUY922 reduced the ALK expression level at 100nM in both crizotinib-resistant cell lines, i.e. SK-N-SH and H2228. **B.** NVP-AUY922 treatment did not restore crizotinib—ALK binding in the two crizotinib-resistant cell lines (SK-N-SH and H2228).

2.3.5 β-catenin expression level positively correlates with the crizotinib responsiveness

To further support the concept that β-catenin can influence the ability of crizotinib to bind to ALK, we sought to find out if there is a correlation between the β-catenin expression level and crizotinib responsiveness among various ALK-expressing cell lines published in the *Cancer Cell Line Encyclopedia* (CCLE) project database (35). As illustrated in **Figure 2.12**, it is evident that a high β-catenin mRNA level significantly correlates with the crizotinib sensitivity (i.e. IC_{50}) based on this analysis (Spearman's correlation, R=0.7, p=0.029).

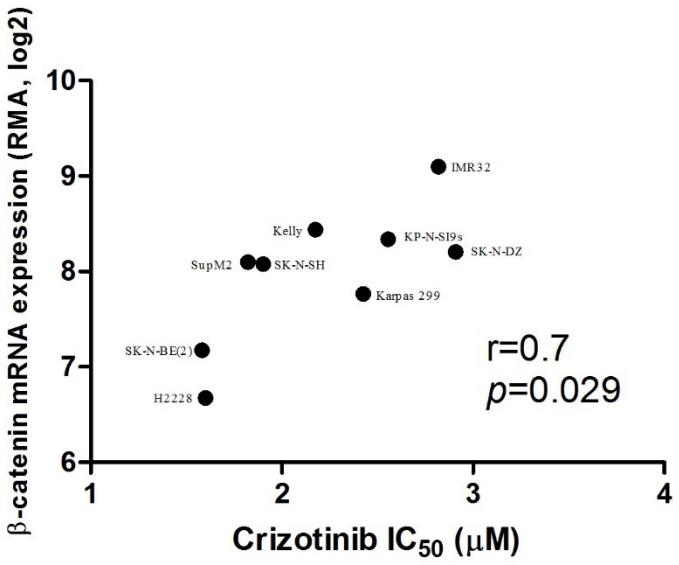

Figure 2.12 Analysis of crizotinib responsiveness amongst various ALK-expressing cell lines showing that crizotinib IC_{50} significantly correlates with the β-catenin expression level.

Cell lines with β-catenin expression level of ≤ 8 were have a crizotinib IC_{50} of ≤ 2μM while Cell lines with β-catenin expression level of ≥ 8 were have a crizotinib IC_{50} of > 2μM (Spearman r =0.7, *p*=0.029). The y-axis represents the β-catenin expression normalized to intensity value of the probe then the data was converted to log2 (RMA, log2) (35). The x-axis represents crizotinib IC_{50} in micromolar (35).

2.3.6 β-catenin physically interferes with crizotinib—ALK binding

In view of our findings that a knockdown of β-catenin can restore crizotinib—ALK binding and the β-catenin expression level significantly correlates with the crizotinib IC_{50}, we hypothesized that the binding between β-catenin and ALK blocks that of crizotinib and ALK. In other words, we predicted that there is a substantial overlap in the ALK binding sites for β-catenin and ALK. Thus, based on the X-ray crystal structure of ALK published by Cui et al. (36), we modeled the crizotinib—ALK binding as well as the β-catenin—ALK binding using ClusPro docking software (Boston University), analyzed and visualized the resulting models using the Molsoft, PyMol as well as Moe software programs. As shown in **Figure 2.13**, β-catenin was predicted to interact with 15 ALK residues, 7 of which (A1126, F1127, G1128, E1129, E1154, V1155 and D1160) reside in close proximity to or surrounding the two ALK residues known to be crucial to the binding of crizotinib, namely a G-rich loop residue (L1122) and a conserved hydrophobic residue (V1130). Additional three residues out of the 15 residues (R1248, R1279 and M1290) were also localized near to another crizotinib-binding residue, D1270. These results suggest that β-catenin binding to ALK will exert substantial impact on crizotinib—ALK binding. Specifically, the presence of β-catenin will likely prevent the crizotinib molecules from reaching the targeted ALK residues or disrupt its binding with certain ALK residues. In keeping with this observation, inhibition of HSP90 did not significantly alter the IC_{50} of crizotinib. We predicted from our modeling and docking procedure that all 12 ALK residues implicated in binding to HSP90 are relatively remote from the ALK residues required for binding crizotinib.

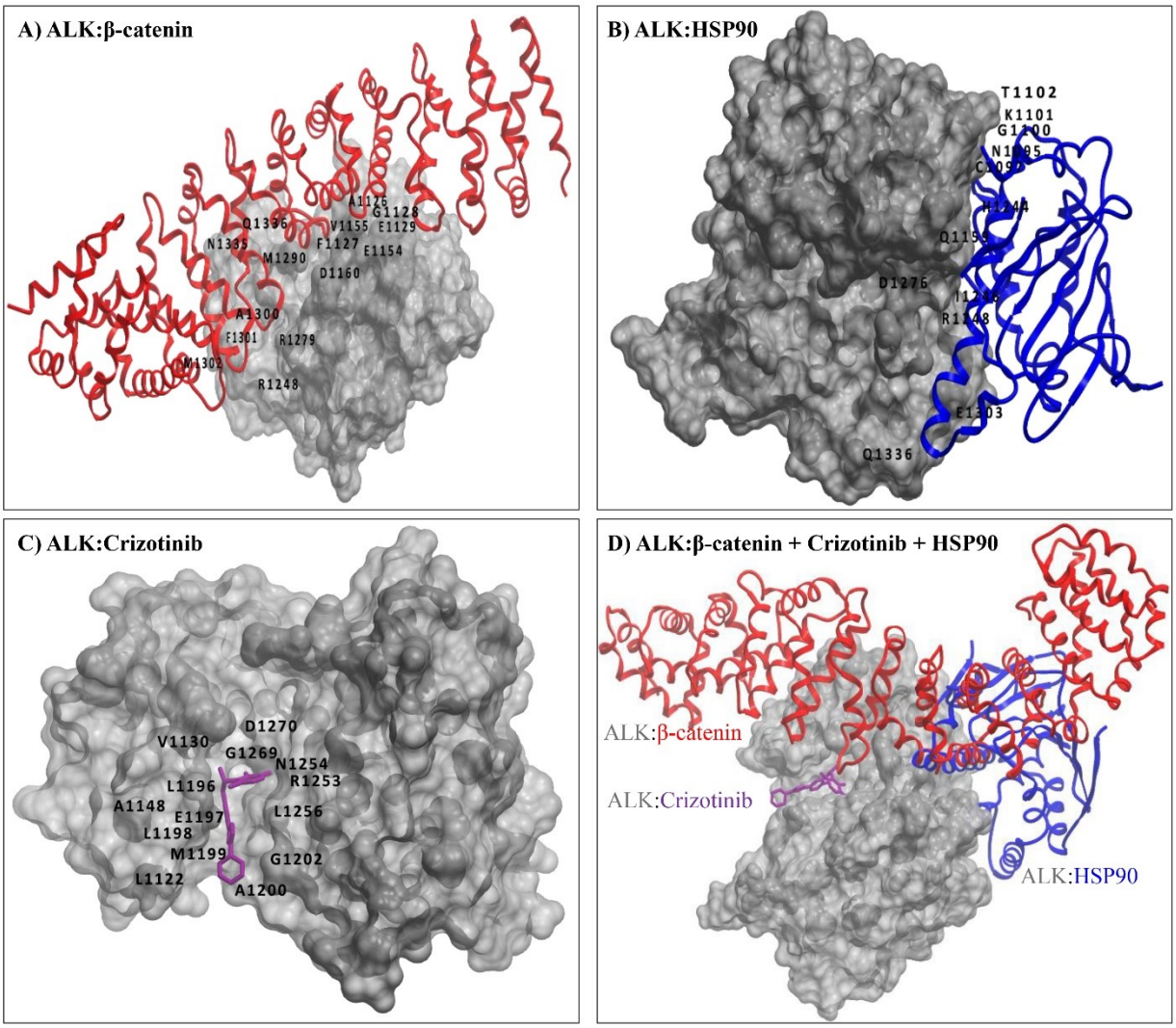

Figure 2.13 Computational analysis of ALK-β-catenin interaction supports the observed blockage of crizotinib—ALK binding.

A. β-catenin was predicted to interact with 15 ALK residues (A1126, F1127, G1128, E1129, E1154, V1155, D1160, R1248, R1279, M1290, A1300, F1301, M1302, N1335 and Q1336). **B.** HSP90 was predicted to interact with 12 ALK residues (N1095, C1097, G1100, K1101, T1102, Q1159, H1244, I1246, R1248, D1276, E1303, and Q1336). **C.** Crizotinib was reported to bind to 14 ALK residues (L1122, V1130, A1148, L1196, E1197, L1198, M1199, A1200, G1202, R1253, N1254, L1256, G1269, and D1270). **D.** Interaction of ALK with β-catenin and crizotinib and HSP90.

2.4 Discussion

The advent and application of specific ALK inhibitors have significantly improved the clinical outcome of patients with ALK^+ tumors, which include (most notably) ALK^+ALCL and ALK^+ lung cancers (8). Crizotinib is the first in the class of ALK inhibitors. In two clinical studies, crizotinib used as a single agent has shown remarkable anti-tumor activity in relapsed ALK^+ALCL patients (19, 20). Unfortunately, based on the results of a number of other clinical studies, resistance to ALK inhibitors occurs relatively frequently (57, 58) (25, 59). While the mechanisms underlying crizotinib resistance is incompletely understood, the acquisition of crizotinib-induced secondary mutations, such as G1202R and G1269A in the ALK kinase domain, is believed to be an important factor (8). In addition to *ALK* mutations, other mechanism of resistance to ALK inhibitors include *ALK* gene amplification and activation of alternative survival signaling pathways such as that of Kras and EGFR (60, 61). Thus far, there are relatively few options available to overcome drug resistance of tyrosine kinase inhibitors. The key strategy has been the development of new generations of ALK inhibitors, with the hope that these drugs can bind to ALK via alternative sites that are not affected by the mutations (8). However, the efficacy of these new inhibitors is not consistent nor predictable (62-64).

Results from this study are in agreement with the previous observation that crizotinib sensitivity is highly variable among ALK^+ human cancer cells (16, 22, 23). For instance, we found that ALK^+ALCL cell lines (Karpas 299 and SupM2) are highly crizotinib-sensitive, as compared to most neuroblastoma cell lines and H2228. It has been reported that the portion of ALK (i.e. in the form of NPM-ALK) expressed by Karpas 299 and SupM2 cells are un-mutated (49). The high crizotinib sensitivity of NB1 also correlates with the previously published data that these cells carry wild-type ALK (albeit amplified) (22, 51, 65, 66). Nonetheless, it is rather puzzling why IMR32 and GOTO, both of which were previously reported to carry wild-type *ALK* (67), are highly crizotinib-resistant, and we believe that results from this current study have shed light to this question.

Using a cohort of 7 ALK^+ cell lines that are highly variable in crizotinib sensitivity (IC_{50} ranging from 10 nM to 1800 nM), we studied the biological basis of crizotinib resistance. An important observation from our studies is that of a significant correlation between the crizotinib sensitivity

and crizotinib—ALK binding. Using 52°C as the cut-off in the CETSA assay, we found that all 3 crizotinib-sensitive cell lines demonstrated crizotinib—ALK binding, in contrast with none of the 4 resistant cell lines, including the two cell lines that carry wild-type ALK (IMR32 and GOTO). Unlike most of the previously published studies of crizotinib resistance, which focused on the correlation between *ALK* mutations and the *in vitro* sensitivity to crizotinib, this current study has provided direct evidence highlighting the importance of the physical interaction between crizotinib and ALK as the key determinant for crizotinib sensitivity. It is perceivable that the interaction between crizotinib and ALK may be modulated by at least 3 major factors: 1) the overall biochemical and biological status that are cell-type specific; 2) the 3-dimensional structure of ALK, which is in turn strongly influenced by the presence of *ALK* gene mutations and its abnormal fusions with other genes; 3) the interactions between ALK and its binding proteins.

To assess the relevance of the overall biochemical/biological status of the cells, we asked if *NPM-ALK* (which exist in the two highly crizotinib-sensitive cell lines, Karpas 299 and SupM2) forced expressed in crizotinib-resistant cell lines can bind crizotinib strongly. If the biochemical/biological status of the cells plays a key role in determining crizotinib—ALK binding, one will expect that this interaction between NPM-ALK and crizotinib will be greatly diminished in the three crizotinib-resistant cell lines. Our observation that NPM-ALK remained to effectively bind to crizotinib at 52°C in the new environment strongly argues against the importance of this factor. Moreover, these experiments also have excluded the possibility that the differential crizotinib—ALK binding is due to substantial differences in the efficiency of the intracellular transport of crizotinib and/or its bioavailability inside the cells. This conclusion is further supported by our observation that increasing the concentrations of crizotinib in the tissue culture did not appreciably affect crizotinib—ALK binding detectable by using CETSA (Figure 3).

There is substantial amount of evidence supporting the importance of the structure of ALK as a determinant of the interaction between ALK and ALK inhibitors. Specifically, *ALK* mutations are known to exist and believed to be a major mechanism of the clinical resistance of ALK inhibitors (8). In the field of ALK^+ALCL, we are aware of only two publications describing ALK mutations in cell lines expressing NPM-ALK, and these mutations do not overlap with the mutations identified in this study (49, 68). In the first study, the author generated crizotinib-resistant clones

derived from Karpas 299 and SupM2, and they identified two *ALK* mutations, namely L1196Q and I1171N (49). The L1196Q mutation found in the Karpas 299 clone is situated at the crucial ATP/crizotinib binding site, and is believed to confer crizotinib resistance by virtue of steric hindrance. The I1171N mutation in the SupM2 clone is located in the vicinity of the kinase activation loop and believed to increase the affinity for ATP at the expense of crizotinib (49). In the second study, a mutation at the residue 1171 (i.e. I1171T) was again found in a crizotinib-resistant clone derived from Karpas 299 (68). In the current study, we also found two mutations at the tyrosine kinase domain of ALK, namely G329A (or G1269A) and G262R (or G1202R) in the resistant clones of Karpas 299 and SupM2, respectively. The clinical significance of these two mutations is substantiated by the observation that they have been found in tumors samples from ALK^+ lung cancer patients (8).

A good number of studies have been previously published in explaining how ALK mutations result in resistance to ALK inhibitors such as crizotinib. As mentioned above and exemplified by the I1171N mutation, one of the mechanisms is related to the relatively high efficiency of ATP recruitment by some ALK mutants, thereby minimizing the inhibitory effect of crizotinib (22, 60). In support of this concept, a study using different EML4-ALK constructs mutated at various sites of the ALK tyrosine kinase domain has concluded that these ALK mutations frequently result in increased ATP-ALK binding and enhance the survival of crizotinib-treated Ba/F3 cells transfected with these EML4-ALK mutants (22, 60). Nonetheless, to our knowledge, direct evidence suggesting that ALK mutations can effectively decrease the binding between ALK and ALK inhibitors is lacking, and our results from studying crizotinib-resistant Karpas 299 and SupM2 clones have provided the first direct evidence. Consistent with our concept that crizotinib—ALK binding is a key determining factor of crizotinib sensitivity, we found ALK gene mutations in both our generated crizotinib-resistant cell clones derived from Karpas 299 and SupM2. Three *ALK* mutations were found in SupM2 and 1 mutation was found in Karpas 299. In support of the clinical relevance of our findings, two of these 4 mutations, both of which are located in the tyrosine kinase domain, have been previously described in crizotinib-resistant ALK^+ lung cancers (8). Using CETSA, we had confirmed that both NPM-ALK mutants do not bind to crizotinib at 52°C.

With respect to the third factor that might regulate the interaction between crizotinib and ALK, we hypothesize that the interaction between ALK and its binding partners may play a key role in influencing crizotinib—ALK binding, and thus, crizotinib-resistance. This hypothesis is based on a number of observations. First, 2 of the 4 crizotinib-resistant cell lines included in this study, namely IMR32 and GOTO, are known to carry wild-type *ALK*. Thus, in addition to gene mutations of *ALK*, there are likely alternative mechanisms to promote crizotinib resistance. Second, it has been published that cell lines carrying the same mutated ALK (e.g. F1174L in Kelly and LAN-1, both of which are neuroblastoma cell lines) displayed drastically different IC_{50} to crizotinib (69). Third, the interacting proteins of oncogenic tyrosine kinases have been shown to modulate resistance to tyrosine kinase inhibitors, although the exact mechanisms are unknown (70-74). With this hypothesis, we made the observation that β-catenin, previously shown to be a binding partner of NPM-ALK (34), is differentially expressed between crizotinib-sensitive and –resistant cell lines. Importantly, siRNA knockdown of β-catenin significantly enhanced crizotinib—ALK binding and the sensitivity to crizotinib in crizotinib-resistant cells (IMR32, SK-N-SH and crizotinib-resistant SupM2 cell clone). Using computation, we have collected evidence suggesting that the binding of β-catenin likely hinders the binding of crizotinib to ALK, and this correlates well with our model. Interestingly, β-catenin has been reported to contribute to drug resistance in many cancer types, including those in which oncogenic tyrosine kinases are not a major driver (71, 74). Another important consideration is that the resistance to crizotinib in cells treated with β-catenin siRNA remained to be relatively high (i.e. IC_{50} ~300 nM). Thus, it is possible that other ALK-interacting proteins (yet to be identified) may continue to hinder the binding of crizotinib to ALK, even in the absence of β-catenin. If these additional ALK-interacting proteins can be identified, simultaneous inhibition of these proteins along with β-catenin may further sensitize these cells to tyrosine kinase inhibitors. This new knowledge may underlie a novel approach in overcoming tyrosine kinase drug resistance. This approach may be particularly useful, considering the observation that siRNA knockdown of β-catenin was found to be effective even in cells with ALK mutations.

CETSA is a recently described method that has been shown to be useful in evaluating the clinical utility of new drugs (75-80). To date, <15 studies using CETA have been published in the literature. Results from this study have strongly suggested that CETSA assay is a useful tool to

predict crizotinib sensitivity in ALK^+ cancers. Compared to many other assays used to assess drug resistance, such as those measuring ATP binding by recombinant oncogenic proteins, the use of CETSA is advantageous in that the drug-target interactions are evaluated in a relevant cellular context. Whether CETSA can be used in the clinical setting to predict drug sensitivity probably needs large-scale studies employing clinical samples.

In conclusion, our study has provided novel insights into the mechanism underling the resistance to crizotinib in ALK^+ cancers. Our studies have provided direct evidence that ALK-crizotinib interaction is the key determinant and predictor of crizotinib sensitivity in these cancer cells. Furthermore, our finding that β-catenin as an ALK-binding protein can substantially contribute to crizotinib resistance has opened a new avenue in overcoming the clinical resistance to tyrosine kinase inhibitors. Lastly, our data has suggested that further investigation of CETSA used in the clinical setting is warranted.

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Chapter 3

High expression of β-catenin confers crizotinib resistance in the small subset of stem-like neuroblastoma cells

This chapter contains a proportion of the manuscript in preparation:

Alshareef A, Gupta N, Zhang HF, Wu C, Lai R. High expression of β-catenin confers crizotinib resistance in the small subset of stem-like neuroblastoma cells. (2017, Submitted).

As first author of this paper, I prepared the first draft and revisions based on the suggestions and comments of the co-authors. I designed and performed most of the experiments described herein, except for the following: Gupta N, Zhang HF and Wu C performed a small portion of experiments, data shown in Figure 3.1 and Figure 3.2, and contributed in finalizing the manuscript. Lai R supervised the whole project. Lai R provided numerous comments and final review of the manuscript before it was submitted for publication.

3.1 Introduction

Neuroblastoma (NB) is the most common extra-cranial malignancy and the leading cause of cancer-related deaths in childhood (1, 2). Despite recent advances in chemotherapy and surgical care, the 5-year survival for patients with high-risk NB is less than 40% (1, 2). It is believed that NB arises from the neuroectodermal precursor cells derived from the neural crest; accordingly, NB tumors are typically found along the sympathetic nervous system chain (3). The clinical courses of NB patients are highly heterogeneous disease, and the most important clinicopathologic parameters used for risk stratification are age at diagnosis, clinical stage and tumor histology (3). Certain genetic alterations including *MYCN* amplification, deletion of the chromosomal region *1p36* and gain of the *17q* region, have been associated with a worse clinical outcome in NB patients (2). Recent studies also have highlighted the importance of intra-tumoral heterogeneity and the existence of cancer stem cells as important contributing factors to treatment failure in NB patients (4). Generally, one of the characteristics of cancer stem cells is their high chemo-resistant property, and it is believed that this phenotype is related to some of their characteristics, including their self-renewal capability, resistance to DNA damage/apoptosis, a poor uptake as well as efficient efflux of cytotoxic drugs (5). In NB tumors, it has been shown that cancer stem cells can be identified and purified by virtue of their expression of a number of markers, with CD133 and nestin most frequently used (6, 7).

Anaplastic lymphoma kinase (ALK), which encodes a tyrosine kinase, was initially discovered and characterized in anaplastic large cell lymphoma (ALCL) carrying the characteristic reciprocal chromosomal translocation, *t(2;5)* (8). In normal cells, the expression of ALK is restricted to embryonic neuronal cells where activation of this tyrosine kinase promotes cell proliferation, survival and differentiation (9). In NB cells, where ALK expression is frequently found, many studies have provided evidence that ALK also promotes cell growth (3). In support of its importance, a high expression of ALK detectable by immunohistochemistry has been reported to significantly correlate with a poor clinical outcome in NB patients (10-13). Nonetheless, the pathogenetic role of ALK in these tumors remains to be incompletely understood. To date, the oncogenic role of ALK in NB cells is best defined by studying the biological effects of specific *ALK* mutations localized in its tyrosine kinase domain (14-17). In this regard, three mutation sites

present in the tyrosine kinase domain (i.e. 1174, 1245 and 1275) were found to account for 85% of all *ALK* missense mutations in NB (18). The oncogenic potential of ALK^{F1174L} has been the most studied, as this *ALK* mutant was found to exert potent oncogenic effects in both *in vitro* and *in vivo* models (19). In keeping with the importance of this *ALK* mutation, patients with tumors carrying *ALK* mutation at residue 1174 were found to have a poor clinical outcome (18). In view of these observations, crizotinib, the first ALK inhibitor approved for clinical use, was tested to treat NB patients with recurrent or refractory diseases in a phase 1 clinical trial (20). Unfortunately, the overall clinical response to crizotinib was suboptimal. Specifically, only 2 out of 34 (6%) patients showed complete remission and 8 (23.5%) showed stable disease, however, 24 (71%) showed progressive disease (20). In fact, this clinical observation correlates with the results of several *in vitro* studies, which found that NB cell lines display a wide range of crizotinib sensitivity, with the IC_{50} (i.e. inhibitory concentration at 50%) ranging from 10 to >3000 nM (18, 21, 22). With respect to ALK^{F1174L}, it has been shown that this specific mutation can increase the affinity for ATP at the expense of crizotinib (18), but ALK^{F1174L}-carrying cell lines displayed drastically different IC_{50} to crizotinib (i.e. IC_{50}, 400 to 2000 nM) (23). Overall, the mechanism underlying the crizotinib resistance in NB cells is incompletely understood.

We have recently published evidence that the physical interaction between ALK and crizotinib is an important determinant of crizotinib sensitivity in NB cells, and this interaction may be affected by the mutational status of *ALK*. Furthermore, β-catenin, a binding partner of ALK in NB cells, can provide significant hindrance to the crizotinib—ALK binding, especially when it is highly expressed (21). In this study, we asked if these observations hold true for cancer stem-like cell population in NB. Stem-like NB cells were purified based on their responsiveness to a Sox2 reporter, a strategy previously used for several different cancer models (24-29).

3.2 Materials and Methods

3.2.1 Cell lines and stable cell clone generation

Details about the two neuroblastoma cell lines, GOTO and SK-N-SH, have been described in

Section 2.3.1 (Chapter 2). The RU and RR cells derived from the GOTO and SK-N-SH cell lines were generated as previously described (26, 27). Briefly, these cells were infected with lentivirus carrying the pGreenFire1-mCMV-EF1-Puro vector or pGreenFire1-mCMV-Sox2SRR2-EF1-Puro vector (SBI System Biosciences, CA, USA). The pGreenFire1-Sox2SRR2-mCMV-EF1-Puro vector contained three tandem repeat of Sox2 regulatory region 2 (SRR2), which is 5'-AAAGAATTTCCCGGGCTCGGGCAGCCATTGTGATGCATATAGGATTATTC-ACGTGGTAATG-3'. The underlined sequence is the Sox2 consensus sequence. Stable cell clone were selected for two weeks in media containing 2μg/ml puromycin. To isolate the RR and RU cell clones, ~10% cells showing the highest/lowest GFP were isolated respectively using fluorescence activated cell sorting (FACS) as described previously (26, 27). All the stable cell clones derived from these cells were cultured in the same type of medium that was used for the parental cell line. Parental cells and stable cell clones have been authenticated using short tandem repeat DNA profiling (from TCAG Genetic Analysis Facility, Toronto, CA).

3.2.2 Neurosphere formation assay

After trypsinization, single-cell suspensions were obtained by filtering the cells using a 40μm Cell Strainer (BD Biosciences). Then, cells were counted and 500 cells were grown in NeuroCult complete media consisting of NeuroCult Neural Stem Cell (NSC) Basal medium, 1/10 NeuroCult NSC Proliferation supplements, 20 ng/ml EGF, 10 ng/ml bFGF, and 2 μg/ml Heparin. NeuroCult media, supplements, and growth factors were all purchased from Stem Cell Technologies (Vancouver, BC, Canada). Cells were cultured for two weeks before the neurosphere containing more than twenty cells were counted.

3.2.3 RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from RU and RR cell subsets with the RNeasy Mini Kit (Qiagen, Valencia, CA). Trace DNA was removed by treatment with TURBO DNA-free Kit (Ambion, Life Technologies, Carlsbad, California, USA). Reverse Transcription (RT) reactions were performed with 1 μg of total RNA using Superscript First-Strand Synthesis System Kits (Invitrogen, Carlsbad, CA). The sequences of the primers used in this study include: Primer set for *CD133*

(*PROM1*): (F-AGTCGGAAACTGGCAGATAGC) & (R-GGTAGTGTTGTACTGGGCCAAT), primer set for *nestin* (*NES*): (F-GTGGCTCCAAGACTTCC) & (R-GCACAGGTGTCTCAAGG), primers set for the SRR2: (F- ACATTGTACTGGGAAGGGACA) & (R-AGCAAGAACTGGCGAATGTG) and primer set for *GAPDH*: (F-GGTCTCCTCTGACTTCAACAGCG) & (R- ACCACCCTGTTGCTGTAGCCAA). GAPDH was used as an internal control.

3.2.4 Chemoresistance assay

Cells were plated in 96-well plates, 2000 cells/well. 24h after plating, cells were cultured in medium containing different doses of Doxorubicin (i.e. 0μM, 0.25μM, 0.5μM, 1μM, 2μM and 4μM) or Cisplatin (i.e. 0μM, 1μM, 2μM, 4μM, 8μM and 16μM). Cell viability was measured using the CellTiter 96AQueous One Solution Cell Proliferation Assay (Promega), also known as MTS assay, 3 days after drug treatment according to the manufacturer's protocol. Absorbance at 490nm was measured using a microplate reader (BMG Labtech, Ortenberg, Germany). The inhibitory concentration at 50% (IC_{50}) was calculated by Graphpad Prism (La Jolla, CA).

3.2.4 Cellular thermal shift assay (CETSA)

Details about CETSA have been described in Section 2.3.2 (Chapter 2).

3.2.5 siRNAs and plasmids transfection

siRNAs targeting β-catenin (smart pool) as well as a scrambled siRNA control (Dharmacon) were transfected with Lipofectamine RNAiMAX Reagent (Life Technologies) at a final concentration of 40 nM. Human β-catenin and ΔN47 β-catenin pcDNA3 were gifts from Eric Fearon (Addgene plasmids # 16828 and #19287, respectively). The plasmid carrying *NPM-ALK* was a kind gift from Dr. S Morris, St. Jude Children's Research Hospital (Memphis, TN, USA).

3.2.6 Western blot assay

Details about Western blot assay have been described in Section 2.3.5 (Chapter 2).

3.2.7 Statistical analysis

Details about Statistical analysis have been described in Section 2.3.9 (Chapter 2).

3.3 Results

3.3.1 Identification of two cell subsets based on their differential response to a Sox2 reporter

Using a commercially available lentiviral Sox2 reporter, we have previously identified novel intra-tumoral heterogeneity in various types of human cancer (24-27). In this study, we asked if the same intra-tumoral heterogeneity can be identified in NB cells. As shown in **Figure 3.1A**, reporter responsive (RR) cells, which were detectable by flow cytometry based on their GFP expression, were identified in both NB cell lines, GOTO and SK-N-SH, respectively. Based on cells transfected with the empty vector (labeled as *mCMV*) to set the cut-offs, we were able to identify 13.6% and 21.1% for GOTO and SK-N-SH, respectively.

To further study the biological significance of this intra-tumoral heterogeneity, we purified RR cells and RU (i.e. reporter unresponsive) cells derived from both cell lines using a flow cytometric cell sorter, and these subsets were cultured separately. The differential GFP expression levels between RU and RR cells in both NB cell lines are evident in **Figure 3.1B**. As shown in **Figure 3.1C**, purified RU and RR cells derived from these two cell lines had no significant difference in the growth rate. We also confirmed that the gene copy number of the Sox2 reporter integrated into these 2 cell subsets was not significantly different, and thus, the difference in their reporter response was genuine (**Figure 3.1D**). Lastly, since RR cells were found to lose GFP expression gradually (i.e. approximately 25% in 4 weeks), we purified RR cells immediately before each of the following experiments. RU cells remained to be GFP-negative and no further purifications were required.

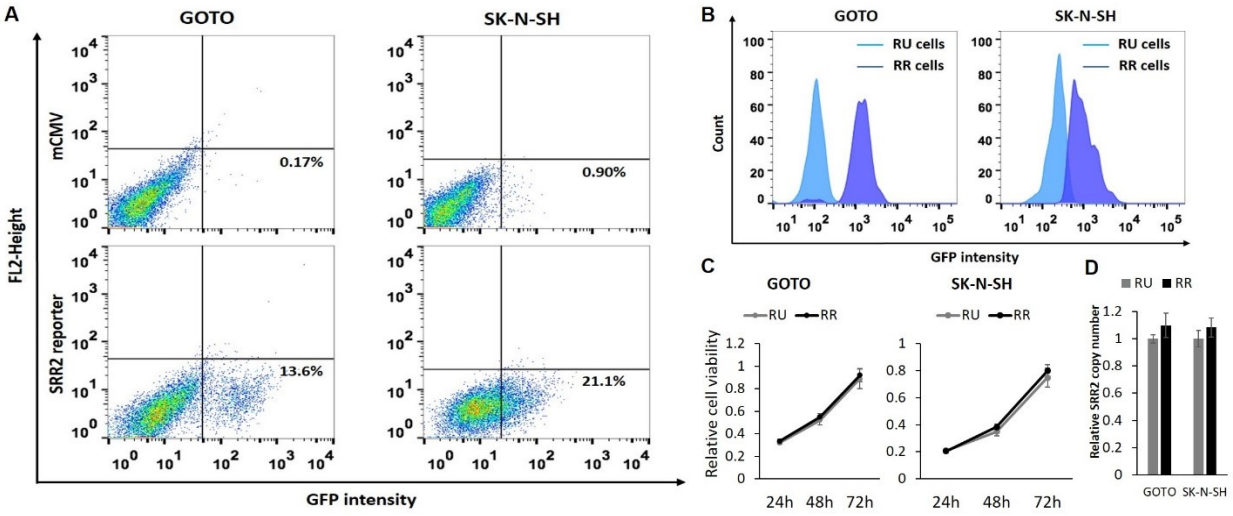

Figure 3.1 Identification of two cell subsets in NB cell lines.

A. FACS dot plots analysis was performed to identify the reporter responsive (RR) cells and reporter unresponsive (RU) cells in NB cells stably infected with the SRR2 reporter. GOTO and SK-N-SH cells infected with the mCMV control lentivirus were used as a negative control for the gating of RU and RR cells. **B.** GFP expression was measured by FACS in RU and RR cells isolated from cells stably infected with the lentiviral SRR2 reporter. **C.** Cell growth rates of RU and RR cells were assessed by MTS assay. **D.** The DNA copy number of the SRR2 reporter integrated in RU and RR cells was measured by real-time PCR. All data are presented as mean ± SD, N=3, **p* <0.05, Student's *t* test. Abbreviations: NB, neuroblastoma; FACS, Fluorescence-activated cell sorting; SRR2, Sox2 regulatory region 2; mCMV: Murine Cytomegalovirus; GFP: Green Fluorescence Protein.

3.3.2 RR cells are more stem-like and chemo-resistant than RU cells

To assess the biological significance of the identified RU/RR dichotomy, we performed a number of functional assays to compare RU and RR cells. First, we compared these two cell subsets with respect to their cancer stem-like features using the neurosphere formation assay. As shown in **Figure 3.2A** and **3.2B**, we found that RR cells showed a significantly higher capacity to form neurospheres than RU cells (~3 fold, $p<0.005$) in both NB cell lines. Correlating with this finding, RR cells purified from both cell lines contained a significantly higher proportion of cells expressing *nestin* and *CD133,* as measured by using quantitative RT-PCR (**Figure 3.2C** and **3.2D**). Of note, both nestin and CD133 have been described as putative cancer stem cell markers for NB cells (30).

We then compared the sensitivity of RU and RR cells to doxorubicin and cisplatin, two chemotherapeutic agents used clinically to treat NB (31). We found that RR cells derived from both cell lines showed a significantly higher IC_{50} (inhibitory concentration at 50%) to doxorubicin than RU cells (GOTO, 2 μM versus 0.71 μM, $p<0.001$; SK-N-SH, 1.2 μM versus 0.51 μM, $p<0.001$). Similarly, RR cells showed a significantly higher IC_{50} to cisplatin compared to RU cells (GOTO, 11.6 μM versus 5.8 μM, $p<0.001$; SK-N-SH, 8.6 μM versus 4.1 μM, $p<0.001$) (**Table 3.1**).

Next, we compared RU and RR with respect to their resistance to oxidative stress, which is known to be higher in normal stem cells and CSCs (32). By subjecting RU and RR cells to increasing concentrations of H_2O_2, a potent inducer of oxidative stress (32), we found that RR cells were significantly more resistant than RU cells (IC_{50} for GOTO, 319 μM versus 192 μM, $p<0.001$; IC_{50} for SK-N-SH, 495 μM versus 185 μM, $p<0.001$) (**Table 3.1**). Taken together, RR cells were more stem-like and chemo-resistant compared to RU cells.

Table 3.1 RR cells are more chemo-resistant than RU cells

Agent Cell line	IC_{50} in μM (standard deviation)		*P* value
	RU	RR	
Doxorubicin (72h)			
GOTO	0.71 (± 0.04)	2 (± 0.09)	< 0.001
SK-N-SH	0.51 (± 0.05)	1.2 (± 0.1)	< 0.001
Cisplatin (72h)			
GOTO	5.8 (± 0.43)	11.6 (± 0.65)	< 0.001
SK-N-SH	4.1 (± 0.33)	8.6 (± 0.75)	< 0.001
H_2O_2 (24h)			
GOTO	192 (± 12)	319 (± 22)	< 0.001
SK-N-SH	185 (± 7)	495 (± 33)	< 0.001

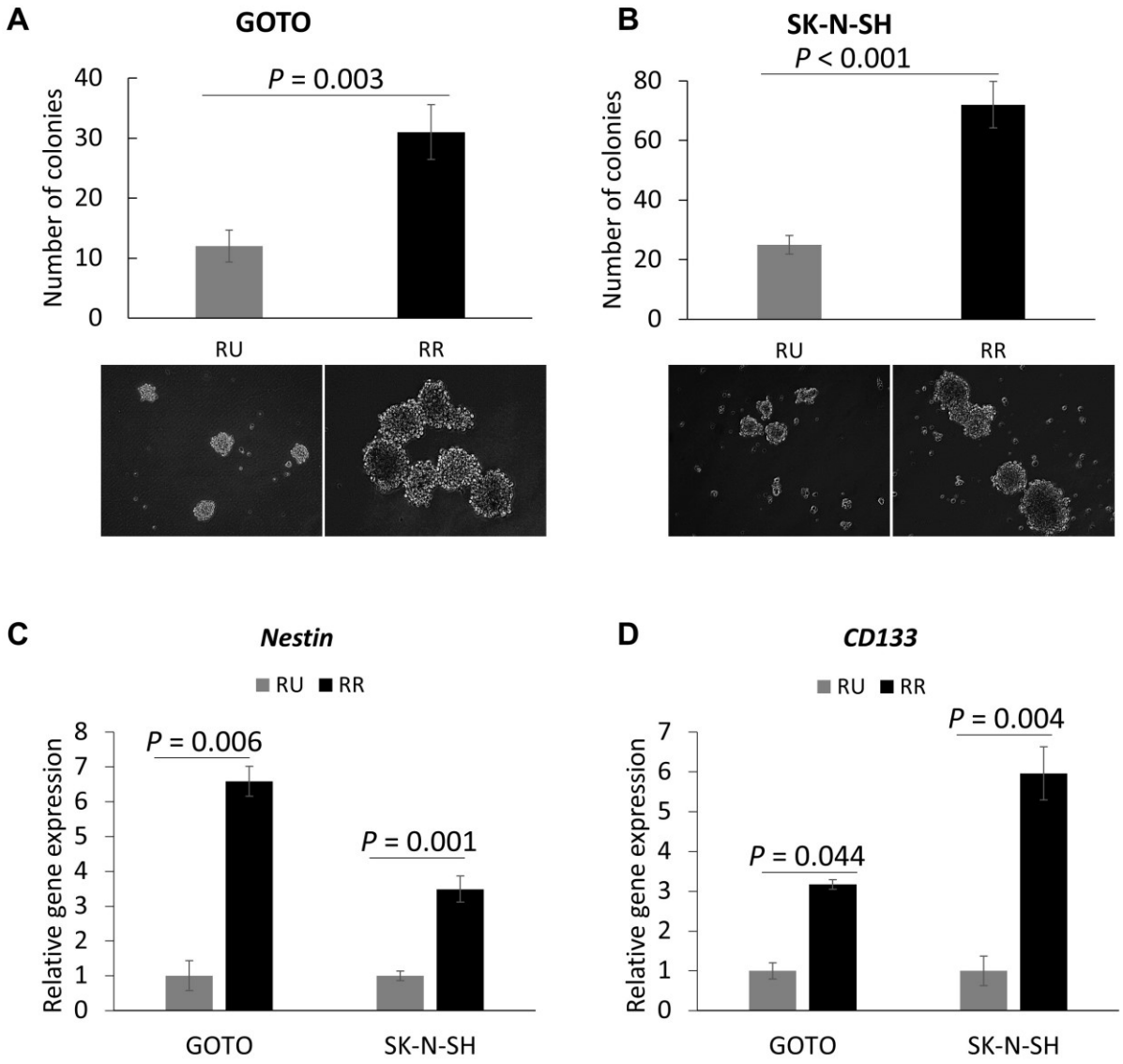

Figure 3.2 RR cells carry more stem-like features compared to RU cells.

A and B. Neurosphere formation assay was performed to compare the CSCs-like properties between RU and RR cells. Images were taken at ×100 magnification. **C and D.** *Nestin* and *CD133* mRNA expressions in RU and RR cells were examined using quantitative real-time PCR. All data are presented as mean ± SD. N=3, Student's *t* test was performed.

3.3.3 RR cells are resistant to crizotinib, an ALK inhibitor

ALK has been recognized as one of the most promising druggable targets identified in NB (33). Thus, we assessed if RR and RU cells have differential sensitivity to crizotinib, an ALK inhibitor (33). RR cells were found to be significantly more resistant to crizotinib than RU cells, with the IC_{50} for RR and RU cells being significantly different (GOTO, 1989 nM versus 988 nM, $p< 0.001$; SK-N-SH, 724 nM versus 452 nM, $p<0.001$) (**Table 3.2**). Since we have recently published that the crizotinib sensitivity in NB cells is linked to the crizotinib—ALK binding (21), we performed cellular thermal shift assay (CETSA), a method shown to be useful in quantifying drug-target interactions (34-36). As detailed previously (21), the crizotinib—ALK binding was assessed 'strong' if crizotinib-treated cells contain a significantly higher level of ALK protein than DMSO-treated cells (i.e. negative control) at 52°C. As shown in **Figure 3.3A**, using Western blots, we identified strong crizotinib—ALK binding in RU cells at 52°C, at which no substantial crizotinib—ALK binding was identified in RR cells. These findings correlate well with our observation that RR cells were more resistant to crizotinib than RU cells.

Table 3.2 RR cells demonstrate higher crizotinib IC_{50} compared to RU cells

Cell line		Crizotinib IC_{50} in nM for 72h (SD)	*P* value
GOTO	RU	988 (± 107)	0.0007
	RR	1989 (± 149)	
SK-N-SH	RU	452 (± 34)	0.001
	RR	724 (± 46)	

3.3.4 β-catenin contributes to the differential crizotinib sensitivity between RU and RR cells

We have previously shown that the β-catenin—ALK interaction contributes to the weak crizotinib—ALK binding and increases the crizotinib resistance in NB cells (21). Therefore, we asked if the β-catenin—ALK interaction also plays a role in generating the differential crizotinib sensitivity between RU and RR cells. As shown in **Figure 3.3B**, RR cells showed a substantially higher β-catenin protein level compared to RU cells. In contrast, the ALK protein level was not appreciably different between RU and RR cells in both NB cell lines. By immunoprecipitation, we found that the β-catenin—ALK interaction was more abundant in RR cells (~5-fold) relative to RU cells (**Figure 3.3C**).

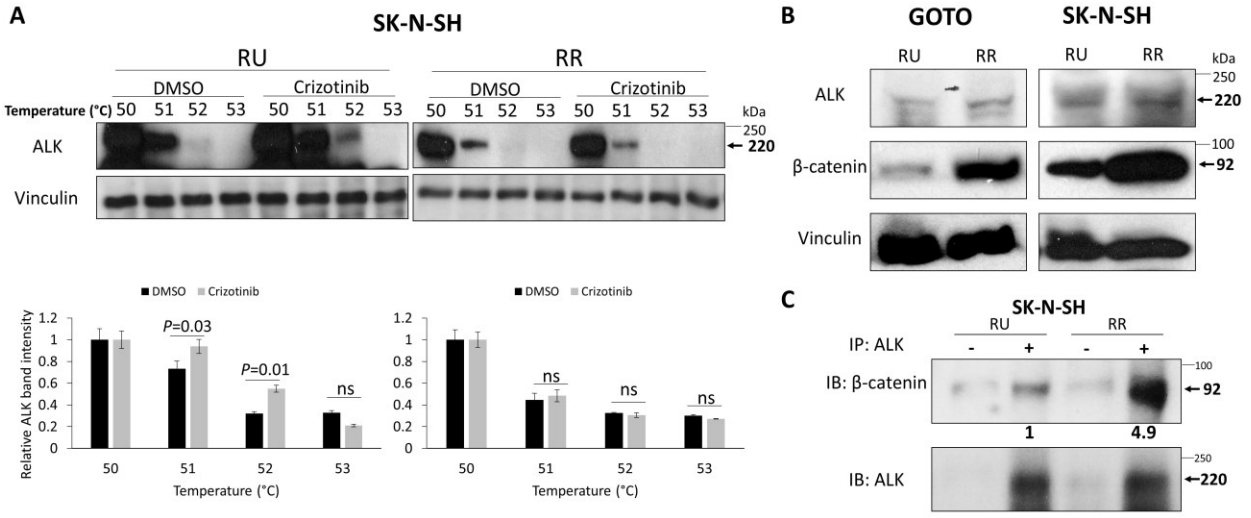

Figure 3.3 RR cells demonstrate no crizotinib—ALK binding and higher expression level of β-catenin compared to RU cells.

A. CETSA was performed to compare crizotinib—ALK binding ability between RU and RR cells. RU and RR cells derived from SK-N-SH were treated with DMSO or 500 nM crizotinib for 6 hours. Representative ALK blots are shown on the upper panel, vinculin was included as a loading control in this experiment. All data are presented as mean ± SD. N=3, Student's *t* test was performed. **B.** The expression of ALK and β-catenin protein in RU and RR cells were measured by Western blot. Vinculin level was blotted as a loading control. **C.** Co-immunoprecipitation assay was performed to pull-down ALK, without ALK antibody (-) or with ALK antibody (+), and the result showed that a substantial amount of β-catenin can be pulled down by ALK in RR cells but not in RU cells.

To better define the link between the β-catenin—ALK interaction and the resistance to crizotinib in NB cells, we transfected RU cells purified from SK-N-SH with a *β-catenin* mutant that carries a deletion of 47 amino acids in the N-terminal region (ΔN47). The logic for deleting this particular segment of β-catenin is based on the results of our prior computational analysis of the β-catenin—ALK interaction (21). Specifically, we predicted a total of 16 residues of β-catenin are involved in the β-catenin—ALK interaction, as illustrated in **Figure 3.4A.** By deleting the first 47 amino acid residues in the N-terminal of β-catenin, we essentially removed 25% of the total β-catenin residues involved in the binding. Furthermore, the deletion of the N-terminal was chosen because it is farthest away from the β-catenin DNA binding domain, as illustrated in **Figure 3.4A**. As shown in **Figure 3.4B**, RU cells transfected with *β-catenin-wt* expressed a relatively high level of β-catenin by Western blots. By immunoprecipitation, evidence of the β-catenin—ALK binding was readily identifiable in these cells. In RU cells transfected with ΔN47, β-catenin—ALK binding was also observed, however, the pulled down ALK was substantially higher in the RU cells transfected with ΔN47.

Using CETSA, we then examined the crizotinib—ALK binding in RU cells transfected with the empty vector, *β-catenin-wt* and ΔN47. As shown in **Figure 3.4C**, upon the enforced expression of β-catenin-wt, the crizotinib—ALK binding was largely abrogated. Importantly, compared to the empty vector, ΔN47 expression did not completely abrogates the interaction between crizotinib and ALK. Thus, this mutant would be unable to appreciably interfere with the crizotinib—ALK interaction. In keeping with this concept, we found that the IC_{50} of cells transfected with *β-catenin-wt* increases approached that of native RR cells (i.e. 724 nM); in comparison, the IC_{50} of cells transfected with ΔN47did not change significantly (**Figure 3.4D**).

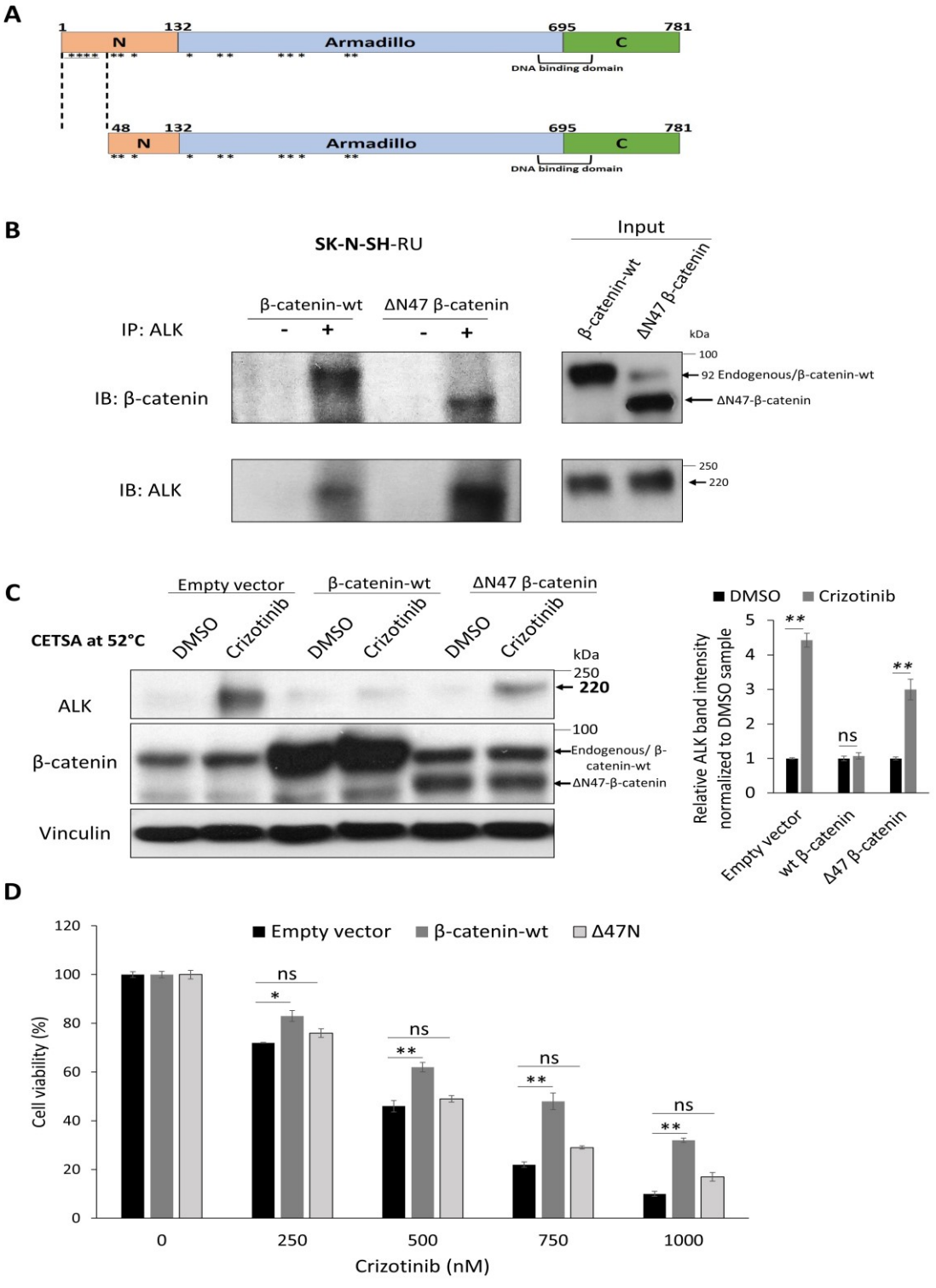

Figure 3.4 Enforced β-catenin-wt interact with ALK and abrogate crizotinib—ALK binding in SK-N-SH-RU cells.

A. Diagram of the two *β-catenin* plasmids used in this study. Locations of computationally predicted β-catenin residues interacting with ALK (16 residues, *). **B.** ALK pull-down was performed using co-immunoprecipitation assay in SK-N-SH-RU cells that were transfected with either empty vector, β-catenin-wt or ΔN47-β-catenin. Enforced expression of β-catenin-wt/ΔN47-β-catenin showed substantial ALK-β-catenin binding when compared to the negative control. **C.** CETSA was performed to compare crizotinib—ALK binding ability in RU that were transfected with either empty vector, β-catenin-wt or ΔN47-β-catenin. Cells were treated with DMSO or 500 nM crizotinib for 6 hours. Representative ALK Western blots are shown on the left panel. Vinculin level was blotted as a loading control. **D.** Enforced expression of β-catenin-wt but not ΔN47 significantly affects crizotinib sensitivity in RU cells in comparison to empty vector transfected cells. All data are presented as mean ± SD, N=3, *P<0.05, **P<0.01, Student's t test.

3.3.5 Lack of crizotinib—ALK binding is not due to lack crizotinib bioavailability

Since the reduced intracellular availability of a variety of therapeutic drugs, through the increased of efflux or decreased of influx, is a well-known mechanism thereby CSCs lead to its resistance (30), we aimed to assess whether the differential crizotinib—ALK binding among RR cells is due to a difference in the availability of crizotinib in the cells, we transiently transfected *NPM-ALK* into the RR cells. By CETSA, we found substantial crizotinib-NPM-ALK binding (**Figure 3.5A** and **3.5B**). In the same experiments, there was no substantial binding between crizotinib and the native forms of ALK. These results support the concept that the differential crizotinib sensitivity and crizotinib—ALK binding between RU and RR cells is not related to a lack of crizotinib inside the RR cells.

3.3.6 siRNA knockdown of β-catenin restores crizotinib—ALK binding and sensitizes RR cells to crizotinib

Lastly, we subjected RR cells derived from GOTO and SK-N-SH to β-catenin siRNA knockdown for 72 hours, then we performed CETSA assay. As shown in **Figure 3.6A** and **3.6B**, restoration of the crizotinib—ALK binding observed upon β-catenin siRNA knockdown conferred a significant sensitization to crizotinib, with the IC_{50} decreased from 1851 nM (negative control) to 822 nM in GOTO cells (**Figure 3.6C**). Importantly, the IC_{50} of RR cells transfected with β-catenin siRNA was lower than that of native RU cells (i.e. 988 nM). Similar results were observed for SK-N-SH

cells, with the IC_{50} decreased from 804 nM (negative control) to 472 nM in RR cells transfected with β-catenin siRNA, which was comparable to that of native RU cells (i.e. 452 nM) (**Figure 3.6D**). Of note, β-catenin siRNA knockdown alone (for 72 hours) did not significantly affect the cell growth of both cell lines. Of note, we did not observe robust differential activation/expression of STAT3 and AKT between RU and RR cells (**Figure 3.7A**).

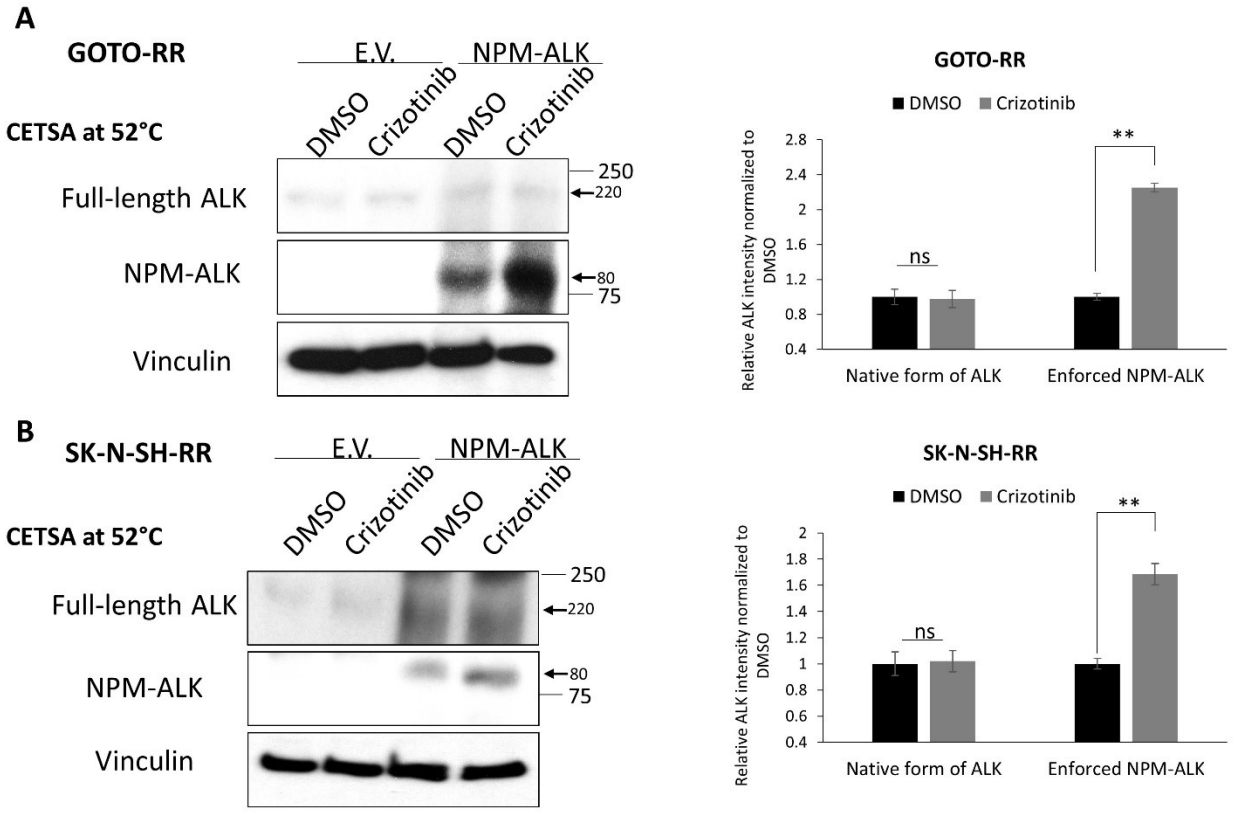

Figure 3.5 Crizotinib resistance in RR cell is not due to lack of intracellular availability of crizotinib.

A and **B.** Enforced expression of NPM-ALK into RR cells derived from GOTO or SK-N-SH showed stabilization of NPM-ALK but not the native ALK. CETSA assay was performed at 52°C. Representative Western blots are shown on the left side and the densitometry quantification data from 3 independent experiments are shown on the right side. Data are presented as mean ± SD. N=3, $^{**}P<0.01$, Student's *t* test.

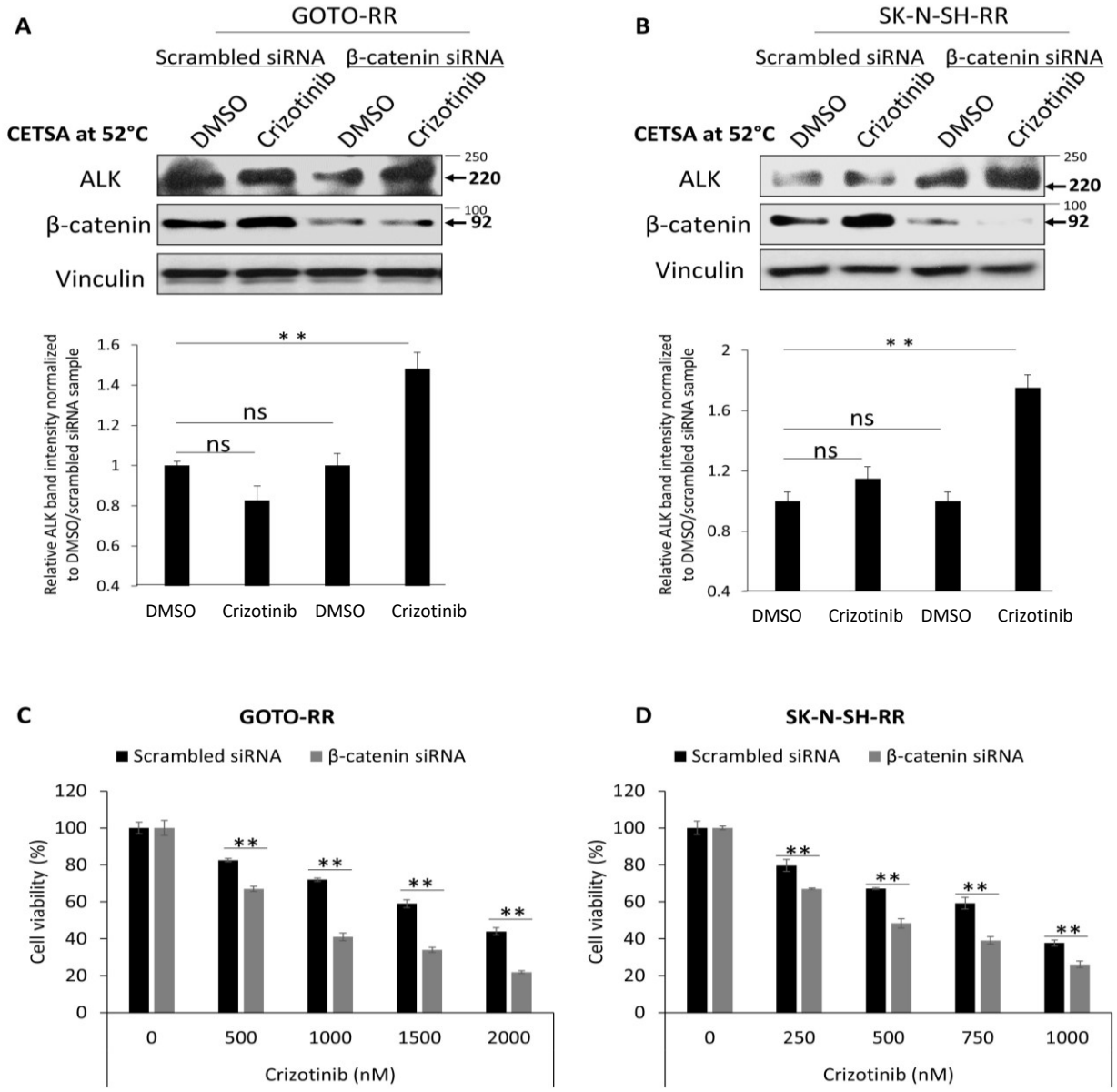

Figure 3.6 β-catenin siRNA knockdown restores the binding between crizotinib and ALK and significantly sensitizes RR cells to crizotinib treatment.

A and **B.** β-catenin siRNA knockdown significantly restores crizotinib—ALK binding upon crizotinib treatment in comparison to scrambled siRNA transfection in RR cells derived from GOTO and SK-N-SH. Representative Western blots are shown on the upper panel and the densitometry quantification data from 3 independent experiments are shown on the lower panel. **C** and **D.** β-catenin siRNA knockdown significantly sensitized RR cells to crizotinib treatment. Data are presented as mean ± SD. N=3, $^{**}P<0.01$, Student's *t* test.

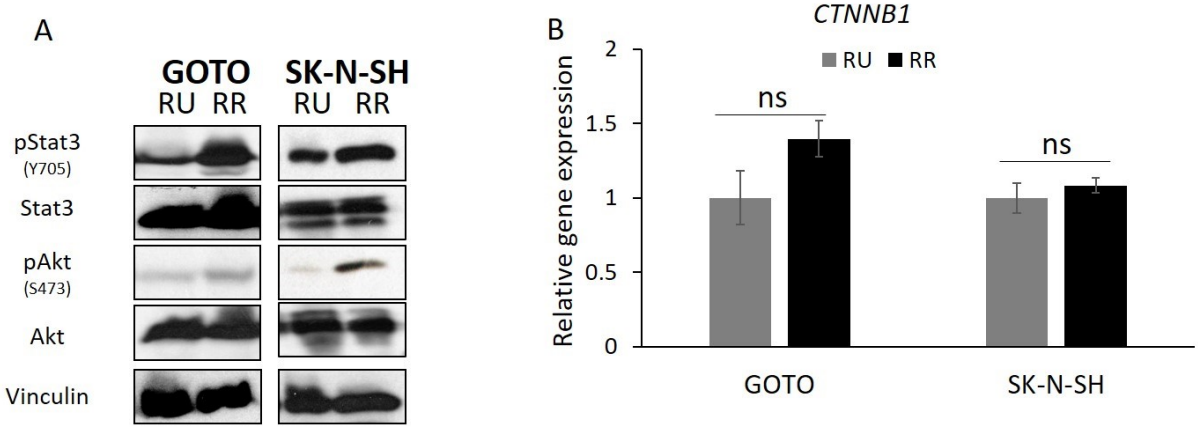

Figure 3.7 RU and RR cells demonstrate no substantial difference for the activation status of Stat3 and Akt as well as β-catenin mRNA level.

A. The activation status of Stat3 and Akt in RU and RR cells were measured by Western blot. Vinculin level was blotted as a loading control. **B.** β-catenin (CTNNB1) mRNA level in RU and RR cells were measured by quantitative real-time PCR. Data are presented as mean ± SD. N=3, ns: not significant, Student's *t* test.

3.4 Discussion

One of our key findings is the identification of the RU/RR dichotomy in NB cells, with the small subset of RR cells being significantly more stem-like than RU cells. This finding is in parallel with the conclusions from several groups, who identified a small subset of CSCs in NB cells (5, 37-45). CD133, a transmembrane glycoprotein, is probably the most frequently used marker used to identify CSCs (5). In other studies, CSCs in NB were identified and/or purified based on their relatively high expression of CD114, Frizzled receptor 6 and ALDH1A2, as well as their ability to exclude Hoechst 33342 (37-45). In contrast with these previous studies, our group identified RR cells based on their responsiveness to the SRR2 reporter. To support the validity of our methodology, we have found evidence that RR cells exhibited significantly higher neurosphere formation ability and chemoresistance than RU cells. Importantly, compared to RU cells, RR cells expressed a significantly higher level of CD133 (5). The link between CD133 and cancer stemness in NB is relatively well supported, as the small subset of CD133-expressing cells are significantly more tumorigenic in mouse xenograft models than their CD133-negative counterparts (38, 41, 46).

It is important to point out that the RU/RR dichotomy identified in NB cells also can be found in other cancer cell types. Specifically, using the same Sox2 reporter (i.e. SRR2), others and we have previously identified intra-tumoral RU/RR dichotomy in estrogen receptor-positive (ER+) breast cancer, triple-negative breast cancer (TNBC), esophageal squamous cell carcinoma and ALK-positive anaplastic large cell lymphoma (25-29, 47). In these study models, RR cells, which consistently represent a relatively small cell subset, were found to be more tumorigenic and stem-like than RU cells. Thus, it is tempting to speculate that the significance of the RU/RR dichotomy and the concept that RR phenotype being a CSCs marker can be generalized to other types of human cancer. While we did not include NB patient samples in the current study, we have documented the existence of the RU/RR dichotomy in tumor samples derived from ER+ breast cancer as well as TNBC (25, 26). Thus, the RU/RR dichotomy is not cell line-specific phenomenon. Overall, we believe that this is a useful experimental model to study the biology of cancer stemness, especially we have demonstrated that the RU and RR phenotype can be converted into each other by using specific experimental manipulation (48, 49).

While CSCs in NB have been shown to be more resistant to conventional chemotherapeutic agents (30), whether they are also more resistant to targeted therapy is largely unknown. Since ALK has been recently postulated to be a useful therapeutic target for NB, we compared the sensitivity of RU and RR cells to crizotinib, the first clinically used ALK inhibitor. We found that RR cells were significantly more resistant to crizotinib than RU cells, suggesting that the existence of cancer stem-like cells in NB cells may be a contributing factor to treatment failure and disease relapses to targeted therapy. To this point, we have identified a small number of published studies that point to a similar conclusion. In one study, it was found that the CSCs in chronic myeloid leukemia cell lines are relatively resistant to imatinib, a tyrosine kinase inhibitor targeting the oncogenic fusion protein BCR–ABL (50). In another study, the CSCs population identified in lung cancer cell lines were found to be relatively resistant to gefitinib, a tyrosine kinase inhibitor targeting the epidermal growth factor receptor (51). In chronic myeloid leukemia, several mechanisms responsible for their resistance to imatinib have been identified. First, CSCs were found to express a relatively high level of BCR-ABL, thereby leading to an increase in IC_{50} (52, 53). Second, the intracellular accumulation of imatinib in CSCs was found to be relatively inefficient, due to the low expression of OCT1 (organic cation transporter-1) (54) as well as the high expression of ABCB1 (ATP Binding Cassette Subfamily B Member-1) (55). Third, CSCs were found to have the preferential activation of pro-survival/anti-apoptotic signaling pathways such as those of MAPK, notch and hedgehog (56). Regarding how RR cells in NB are more resistant to crizotinib, we believe that different mechanisms are in place. Specifically, we did not find a substantial difference in the expression of ALK between RU and RR cells (**Figure 3.3B**). We also found no evidence to support the notion that the intracellular accumulation of crizotinib is suboptimal in RR cells, since transfected *NPM-ALK* expressed in RR cells binds well to crizotinib, as assessed by the CETSA assay. Lastly, other than the robust differential expression of β-catenin, we did not observe a similar robust difference in the expression or activation status of key signaling pathways such as those of STAT3 and AKT (**Figure 3.7A**). Thus, it appears that RR cells in NB utilize different mechanisms to raise their resistance to crizotinib.

To our knowledge, relatively few studies have shown that CSCs derived from various cancer types, including NB, express a relatively high level of β-catenin (38, 57-59). While inhibition of β-catenin was found to decrease cancer stemness and tumorigenicity in CSCs in some of these studies,

whether β-catenin plays a direct role in conferring resistance to conventional chemotherapy or targeted therapy has not been examined. Our findings have led us to believe that β-catenin is a key contributory factor to the differential crizotinib sensitivity between RU and RR cells. Specifically, transfection of *β-catenin* in RU cells conferred resistance to crizotinib, whereas siRNA knockdown of *β-catenin* led to a significant decrease in IC_{50} to the level of RU cells. Regarding how β-catenin mediates these biological effects, results from one of our recently publications have suggested that β-catenin likely hinders the binding of crizotinib to ALK (21). This conclusion is supported by two lines of evidence. First, computational analysis has revealed that the ALK binding site recognized by β-catenin substantially overlaps with that of crizotinib (21). Accordingly, siRNA knockdown of β-catenin in RR cells significantly increased the crizotinib—ALK binding (detectable by CETSA) and decreased the IC_{50}. Second, as shown in this current study, a relatively short deletion in β-catenin (i.e. ΔN47, deletion of the first 47 amino acids out of the total 781 amino acid peptide) abrogated the β-catenin-mediated crizotinib resistance in RU cells (**Figure 3.4C**). Results from the experiments using ΔN47 also argue against the possibility that β-catenin increased the crizotinib resistance simply due to its transcriptional activity, as its DNA binding site is remote from the deletions in ΔN47.

The mechanisms for the different β-catenin expression levels between RU and RR cells is unknown, but some of our observations may have provided some clues. First, we did not observe a significant difference in its mRNA level between RU and RR cells (**Figure 3.7B**), and this finding suggests that the differential β-catenin expression between RU and RR cells is likely due to a post-translational mechanism(s). This concept is supported by a published study showing a substantial difference in the β-catenin protein level between CD133-positive CSCs and CD133-negative bulk cells, despite the fact that their mRNA level of β-catenin was similar between the two cell subsets (60). Second, our recent study of RU and RR cells derived from ALK-positive anaplastic large cell lymphoma cells suggests that differential cytokine stimulation of the Wnt canonical pathway between the two cell subsets may have played a role in this context (48). Specifically, we have found that RR cells expressed more ligands for the Wnt/β-catenin pathway, such as Wnt2B, than RU cells and the co-culture of RR cells with RU cells lead to a dramatic accumulation of the β-catenin in RU cells (48). Therefore, it will be of interest to investigate

whether the different β-catenin expression levels between RU and RR derived from NB cells also occurs in a similar mechanism.

While the SRR2 reporter contains a naturally occurring Sox2 binding consensus sequence present in the enhancer of hundreds of genes in the genome, it proved to represent a useful surrogate marker for the CSCs/RR phenotype and its associated cancer stemness features in multiple cancer types. We now know that, while SRR2 is highly specific to Sox2 in some cancer types such as ER+ breast cancer, we found that SRR2 may be potentially recognized by other stemness-associated transcriptional factors including MYC, STAT3 and NFκB, based on the sequence analysis of the SRR2 (25, 48). More specifically, we have recently showed that SRR2 reporter is regulated by Sox2 and MYC in ALK-positive anaplastic large cell lymphoma cells and by MYC but not Sox2 in TNBC and esophageal squamous cell carcinoma (24, 25, 48). Importantly, the more tumorigenic and stemness features in RR cells were reproducible despite the different SRR2 driver. Furthermore, well-documented CSCs markers, such as CD44, were shown to be significantly increased in RR cells (24, 25, 48). Thus, SRR2 reporter represents a transcriptional signature of this small subset which allowed prospective identification of CSCs.

To conclude, our study has highlighted intra-tumoral heterogeneity in NB cells using a study model that is based on the differential responsiveness to an SRR2 reporter. Although the SRR2 reporter activity is a novel potent marker for the identification of stem-like NB subset for characterization and improved understanding, it does not itself provide a protein for targeted therapies. Our studies have provided novel insights into the mechanism underling crizotinib resistance in NB cells. With the identification of β-catenin, an ALK-binding protein that can substantially contribute to crizotinib resistance in NB cells, we have a direct protein target that could be an important novel NB drug candidate that will maximize the benefit of crizotinib treatment.

3.5 References

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Chapter 4

The absence of a novel intron 19-retaining *ALK* transcript (*ALK-I19*) and *MYCN* amplification correlates with an excellent clinical outcome in neuroblastoma patients

This chapter contains a proportion of the submitted manuscript:

Alshareef A, Irwin M, Gupta N, Zhang HF, Haque M, Findlay S, Rayis M, Al-Dandan S, Lai R. The absence of a novel intron 19-retaining ALK transcript (*ALK-I19*) and *MYCN* amplification correlates with an excellent clinical outcome in neuroblastoma patients. (2017, Submitted).

As first author of this paper, I prepared the first draft and revisions based on the suggestions and comments of the co-authors. I designed and performed most of the experiments described herein, except for the following: Irwin M and Gupta N helped with the statistical analysis shown in Figure 4-12 and Figure 4-13, Zhang HF, Haque M and Findlay S helped with the development of the protocol for *ALK-I19* primer design and detection, Rayis M and Al-Dandan S provided patient's diagnosis, tissue blocks and the clinical data. Lai R provided numerous comments and final review of the manuscript before it was submitted for publication.

4.1 Introduction

Anaplastic lymphoma kinase (ALK), which encodes a tyrosine kinase member of the insulin receptor superfamily, was initially identified as a potent oncogenic driver in anaplastic large-cell lymphoma (ALCL) (1, 2). In ALCL, the tyrosine kinase of ALK is constitutively active, and this is directly resulted from the reciprocal chromosomal translocations fusing the portion of *ALK* that encodes the tyrosine kinase domain and different gene partners (as shown in **Table 1.1**), with the *nucleophosmin* (*NPM*) gene being the most frequently implicated (2). Consequently, multiple cellular signaling pathways and biochemical processes are deregulated, providing the molecular basis underlying the oncogenic effects of ALK (2). Other than ALCL, *ALK* genetic alterations were identified in a variety of tumors including neuroblastoma (NB), myofibroblastoma and non-small cell lung cancer (NSCLC) (3). These genetic alterations include chromosomal translocations involving fusion partners other than *NPM*, chromosomal inversion, gene amplification and activating missense mutations, all of which are believed to result in constitutive activation of the ALK tyrosine kinase and the downstream cellular signaling pathways and biological processes (3). In keeping with the pathogenetic importance of ALK in these human cancers, targeted inhibition of ALK using various tyrosine kinase inhibitors have shown therapeutic efficacy against subsets of ALK-positive tumors (3).

NB is the most common pediatric extra-cranial solid tumor and the survival for metastatic NB remains < 50% despite intensive multi-modality therapies (4). Known prognostic factors at diagnosis that are associated with poor outcome include age of >18 months, *MYCN* amplification, advanced stage, undifferentiated histology and diploid DNA status (5). ALK protein detectable by immunohistochemistry (IHC) has been reported in the majority of NB tumors (6, 7), and it is believed that ALK carries pathogenetic importance in these cells (8-11). *ALK* missense mutations are detected in 8% of NB tumors at diagnosis (12), but the prevalence appears to increase at recurrence (13-15). The three hotspot mutations at residues 1174, 1245 and 1275 within the tyrosine kinase domain account for 85% of *ALK* missense mutations (12). There is increasing evidence that these *ALK* mutations, with the ALK^{F1174L} mutation being the best characterized, are activating and oncogenic *in vitro* and *in vivo* (16). The prognostic significance of *ALK* missense mutations and amplification varies in different studies; one analysis found a significant correlation

between the presence of *ALK*^{F1174L} and *MYCN* amplification (7, 11, 14). Nonetheless, the significance of ALK expression in the absence of *ALK* amplification and/or mutations is not well understood. Furthermore, a previous study reported that ALK protein expression is detectable in NB tumors regardless of the status of *MYCN* amplification (6). Thus, the role and mechanisms of ALK expression in NB need to be further characterized.

In order to achieve a better understanding of the mechanisms that regulate the expression of *ALK* in NB, we examined the mRNA and protein expression in a cohort of NB cell lines and tumors. In particular, we assessed if *ALK* mRNA transcripts in NB cells retain evidence of intron 19 (I19), since ALK transcripts retaining portions of intron 19 were detected in subsets of melanoma and other tumors (8, 17-20), and these intron 19-retaining *ALK* transcripts were found to be responsible for ALK expression in some cases of melanoma (21). Our studies had revealed a unique *ALK* mRNA species in which the entire I19 was included in the mRNA transcripts. The biological and clinical significance of this *ALK* variant, labeled *ALK-I19*, are discussed.

4.2 Methods and Materials

4.2.1 Clinical samples

Human NB samples were collected between September 2006 and May 2014 directly after surgical resection at the Department of Anatomical Pathology of King Fahad Medical City (KFMC), Saudi Arabia. The 37 "biopsy" specimens were pre-chemotherapy (30 NB, 5 GNB and 2 GN). Ten out of the 37 patients had additional samples/tumor resection specimens harvested post-chemotherapy. The cases were selected based on a clear pathological diagnosis, follow-up data, and had not received previous local or systemic treatment. The histological characterization was based on the International Neuroblastoma Pathology Classification (INPC) and the clinicopathological staging was performed in accordance with the International NB Staging System (INSS) (22). Differentiation status was evaluated by two pathologists (S. A. and R. L.). Of note, patients were risk stratified and treated according to the Children's Oncology Group (COG)-risk groups (23). The study was approved by the Institutional Review Board of the KFMC and by the University of

Alberta Research Ethics Board. Cytogenetic studies of the *MYCN* gene amplification status were performed by FISH as described previously (22).

4.2.2 Cell lines and cDNAs

Details about the human cancer cell lines used in this study have been described in Section 2.3.1 (Chapter 2). cDNA templates generated from normal human neonatal brain (catalog # cDNA-hsa-08), normal human fetal lung and spleen (catalog # cDNA-hsa-09), and normal human adult brain, lung and testis (catalog # cDNA-hsa-15) were purchased from Biosettia Inc. (Biosettia, San Diego, CA, USA). cDNA template generated from OVCAR3 cells was a gift from Dr. YangXin Fu (Department of Oncology, University of Alberta). cDNA template generated from BCPAP cells was a gift from Dr. Todd McMullen (Department of Oncology, University of Alberta).

4.2.3 RNA isolation, Reverse Transcription PCR (RT-PCR), quantitative real-time PCR (qRT-PCR), triple-primer-PCR (TP-PCR) and Western blot analysis

Total RNA was extracted from cell lines with the RNeasy Mini Kit (Qiagen, Valencia, CA) and from NB tissue blocks with the PureLink FFPE RNA Isolation Kit (Invitrogen, Carlsbad, CA). Trace DNA was removed by treatment with TURBO DNA-free Kit (Ambion, Life Technologies, Carlsbad, California, USA). Reverse Transcription (RT) reactions were performed with 0.5 to 2 μg of total RNA using Superscript First-Strand Synthesis System Kits (Invitrogen, Carlsbad, CA). Primers were designed using Primer3 software and were purchased from Invitrogen. The sequences of the primers used in this study include:
Primer set #1: (F- ACGTGCTCGGCAATTTACAC) & (R- GGGCCCAGGCTGGTTCATGC)
Primer set #2: (F- AGAAGAAGGCGTCGGAAGTG) & (R- ATGTGCTCAGTTCCCTCCTC)
Primer set #3: (F- AGAAGAAGGCGTCGGAAGTG) & (R- GGGCCCAGGCTGGTTCATGC)
Primer set #4: (F- ACGTGCTCGGCAATTTACAC) & (R- ATGTGCTCAGTTCCCTCCTC)
Primer set #5: (F- CCTGTGGCTGTCAGTATTTGG) & (R- GGACAGGTCAAGAGGCAGTT)
Primer set #6 & #7: (F- GTCTCCTGCATTGTGTCACC) & (R- ATTCAGTCCTGCCTTCCTGC) & (R- TTTTCCGCGGCACCTCCT)
Primer set #8: (F- CCCTGAGTACAAGCTGAGCA) & (R- GGGCCCAGGCTGGTTCATGC)

Primer set #9: (F- CCTCTCTGTGGTGACCTCTG) & (R- TTTTCCGCGGCACCTCCT)
Primer set for GAPDH: (F- GGTCTCCTCTGACTTCAACAGCG) & (R- ACCACCCTGTTGCTGTAGCCAA).
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal control. Sequencing was performed at The Applied Genomics Centre (TAGC), University of Alberta. For Western blot analysis, protein extraction and ALK antibody staining have been described in Section 2.3.1 (Chapter 2).

4.2.4 Immunohistochemistry (IHC)

The immunohistochemical studies were performed using the anti-ALK mouse antibody Ab-1 (Clone 5A4; NeoMarkers) on 4 μm thick paraffin sections at the Cross Cancer Institute, Edmonton, Alberta, Canada. The heat-induced epitope retrieval was performed by heating in Tris–EDTA buffer, pH 9.0. An ALK-translocated t(2;5) anaplastic large-cell lymphoma sample was used as a positive control. Reactive tonsils tissue was used as a negative control. ALK was considered positive when more than 50% of tumor cells were strongly reactive with anti-ALK, and this criterion is similar to that reported previously (6, 24).

4.2.5 Nuclear/cytoplasmic RNA fractionation

Nuclear and cytoplasmic RNAs were isolated using the PARIS Kit (Ambion, Life Technologies, Carlsbad, California, USA) according to the manufacturer's instructions. RNA was quantified, and RT-PCR was performed using ABI (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Trace DNA was removed as described above. Reverse Transcription (RT) reactions were performed with 250 ng of total RNA using Superscript First-Strand Synthesis System Kits (Invitrogen, Carlsbad, CA).

4.2.6 Small interfering RNA (siRNA) transfection, reagents, cell growth analysis and neurosphere formation assay

Four small interfering RNAs (siRNA) species were designed against *ALK-I19*, specifically targeting intron 19, and were purchased from Sigma-Aldrich. The sequences for *ALK-I19* siRNAs are **1.** 5'-AAUCUGAUCACGGUCGGUCCAUU-3' **2.** 5'-AAUUUUGUUCUGGCUUCCA-3' **3.** 5'-AGUGAUAAUGGUCACUCAC-3' **4.** 5'-AAGAUCCCAGCUGCACCCU-3' and their locations in intron 19 are illustrated in **Figure 4.3A**. siRNAs were transfected using Lipofectamine RNAiMAX (Invitrogen). Cell growth was performed as previously described (25, 26). Briefly, cell lines were transfected with either scrambled siRNA or *ALK-I19* siRNA at a final concentration of 40 nM for 72 hours. The cell viability was then measured using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay (Promega, Madison, WI) following the manufacturer's protocol. Details about neurosphere formation assay have been described in Section 3.2.2 (Chapter 3).

4.2.7 Antisense morpholino oligonucleotides

NB cells were transfected with morpholino antisense oligonucleotides using the Electro square electroporator BTX ECM 800 (225 V, 8.5 ms, 3 pulses) (Holliston, MA, USA) according to the manufacturer's instructions. Briefly, 5 million cells were resuspended into 500 µl of media with 1 or 10 µM of either *ALK* exon 19/intron 19 oligonucleotides (5′-GGCTCTGTGCACTCACCAATCATG -3′ or a standard control oligonucleotides and electroporated with the BTX ECM 800. Cells were harvested after 48 hours for RNA and protein extraction.

4.2.8 Statistical analysis

The statistical analyses were performed using either the SPSS V.13.0 statistical software package or the Graphpad Prism6 to compute statistical significance in NB patient groups. Overall survival and disease-free survival curves were plotted using the Kaplan–Meier method and compared using the log-rank test. Student two-tailed test or two-tailed Fisher's exact test were used to test for significant differences between two group means and 2x2 correlations, respectively. *P* values were considered statistically significant at less than 0.05.

4.3 Results

4.3.1 Expression of *ALK-I19* in NB cell lines

We were particularly interested in the intron 19 region (I19) of *ALK*, since portions of I19 have been found in the mRNA transcripts of two *ALK* fusion genes, *EML4-ALK* and *PPFIBP1-ALK*, detectable in lung cancer and myofibroblastoma, respectively (8, 17-20). Of note, these I19-containing *ALK* transcripts have not been fully characterized and their significance is largely unknown. In a subset of melanoma, portions of I19 also was also detected in the *ALK* transcripts; importantly, these I19-containing *ALK* transcripts were found to be responsible for the aberrant expression of ALK in these tumors, as the I19 portions contain the transcription initiation sites (21). To detect I19 in NB, we employed RT-PCR and an array of primer sets specifically designed to detect I19 (**Table 4.1)**. Four NB cell lines, including NB1 (with amplified *ALK*), IMR32 (wild-type *ALK*), GOTO (wild-type *ALK*) and SK-N-SH (mutated ALK^{F1174L}) were included in this study.

Using primer set #1 (spanning from the 5' end of I19 to the 3' end of exon 29), we found an amplicon of ~2,800 bp in 4 of 4 cell lines (**Figure 4.1A**). The size of this amplicon matched that was expected for the combined length of all of the exons (i.e. exon 20 to exon 29) and the portion of I19 spanned by this primer set (i.e. 2,884 bp). Sequencing of this amplicon confirmed this. Triplicate experiments were run and the only one amplicon of ~2,800 bp was consistently found in all 3 runs.

Table 4.1 Primers used in this study

Primer set #	Primer location	Expected product (bp)	Figures	Product	Method
1	Intron 19 to Exon 29	2884	4.1A, 4.2A, 4.3B	*ALK-I19*	RT-PCR
2	Exon 1 to Intron 19	3436	4.1A	*ALK-I19*	RT-PCR
3	Exon 1 to Exon 29	4286	4.1A, 4.3B	*FS-ALK*	RT-PCR
4	mid-point of Intron 19	99	4.1B, 4.4A	*ALK-I19*	qRT-PCR
5	Exon 24 to Exon 25	79	4.1B, 4.4B	Total *ALK*	qRT-PCR
6	Exon 18 to Intron 19	172	4.1C, 4.4D, 4.5A, 4.5B	*ALK-I19*	RT-PCR
7	Exon 18 to Exon 20	289	4.1C, 4.4D, 4.4E	*FS-ALK*	RT-PCR
8	Exon 20 to Exon 29	1636	4.2A	Total *ALK*	RT-PCR
9	Exon 19 to Exon 20	236	4.5A, 4.5B	*FS-ALK*	RT-PCR

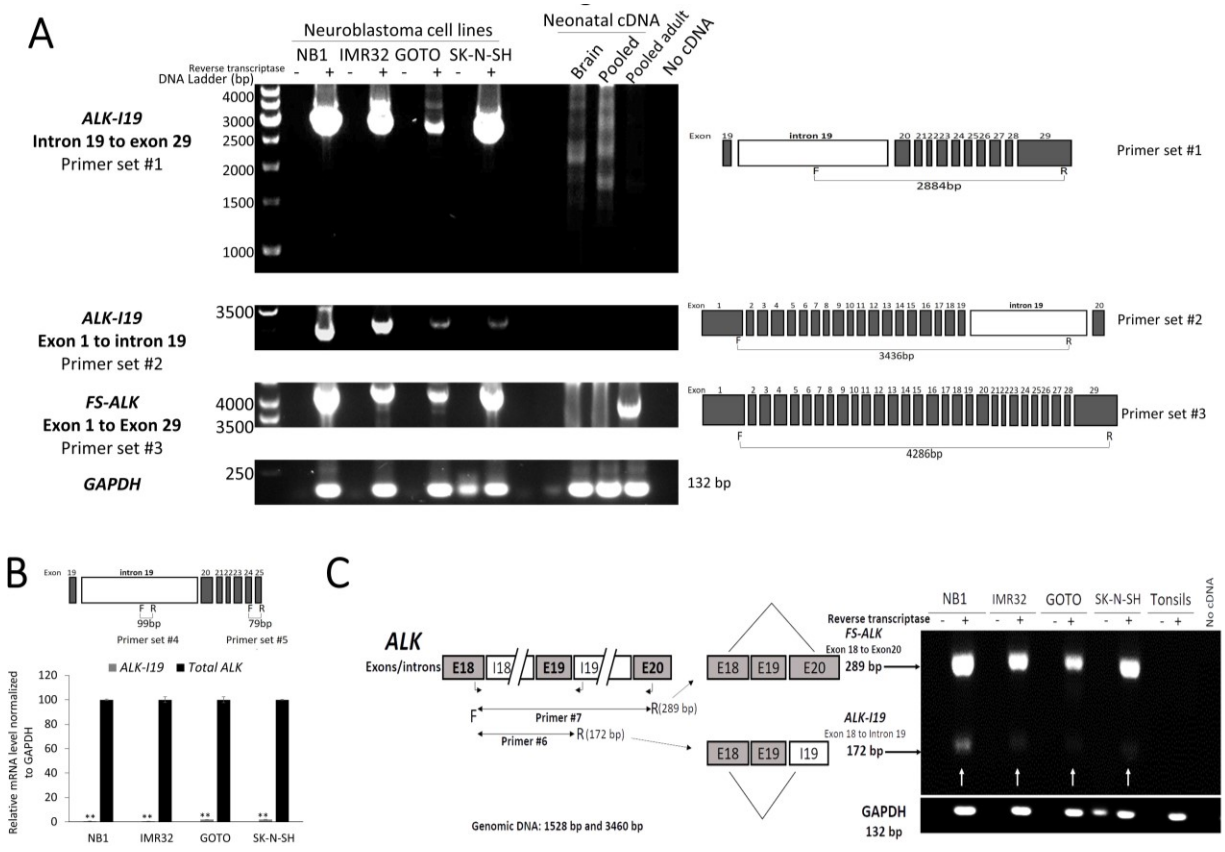


Figure 4.1 Expression of *ALK-I19* in NB cells.

A. Detection of *ALK-I19* and *FS-ALK* transcripts in 4 NB cell lines (NB1, IMR32, GOTO and SK-N-SH) using RT-PCR and primer set #1-3. cDNA samples derived from neonatal brain, pooled normal adult tissues and pooled neonatal tissues were negative for *ALK-I19*. Triplicate experiments were performed and only one amplicon was identified in all runs. GAPDH served as the loading control. **B.** *ALK-I19* was found to be expressed at a significantly lower level when compared to *FS-ALK*, as revealed by quantitative RT-PCR. Primer set #4 was designed to detect *ALK-I19* whereas primer set #5 was designed to detect all *ALK* transcripts. Data are presented as mean ± standard deviation and statistical analysis was performed using Student's *t* test, n=3. The *p* value for all runs is <0.01. **C.** RT-PCR using both primer set #6 (to detect *ALK-I19*) and #7 (to detect *FS-ALK*) generated predominantly the *FS-ALK* amplicons, and this finding further supports the concept that *FS-ALK* is more abundant than *ALK-I19*. GAPDH was used as the loading control. Samples without added reverse transcriptase were used as the negative control.

To confirm the existence of the I19-containing *ALK* transcript (thereafter labeled as *ALK-I19*) in NB cells, we performed RT-PCR using another PCR primer set that spanned from exon 1 to the mid-region of I19 (#2; **Table 4.1**). As shown in **Figure 4.1A**, with the exception of NB1, we found only one detectable amplicon in all cell lines, and the size of ~3,500 bp matched that was expected for the combined length of all of the exons (i.e. exon 1 to exon 19) and the included portion of I19 spanned by the primer sets (i.e. 3,436 bp). Triplicate experiments were run and only one amplicon of ~3,500 bp was consistently detected. Regarding NB1, the amplicons were at ~3,200 bp, since the *ALK* gene in these cells are known to have deletions of exon 2 and 3 (27).

Using both primer set #1 and #2, we did not find evidence of *ALK-I19* in the cDNA samples derived from neonatal brain, pooled neonatal human tissues or pooled adult human tissues; in addition, no evidence of *ALK-I19* was found in a number of ALK-expressing, non-NB human cell lines including Karpas 299 and SupM2 (two ALK-positive anaplastic large cell lymphoma cell lines expressing NPM-ALK), H2228 (a lung cancer cell line expressing EML4-ALK) and U-87 MG (a glioblastoma cell line expressing wild-type full-length ALK)(**Figure 4.2A**). Furthermore, no evidence of *ALK-I19* was found in a number of ALK-negative, non-NB human cell lines including Jeko (a mantle cell lymphoma cell line), A459 (a lung cancer cell line), MCF7 (an ER^+ breast cancer cell line), MB-MDA-231 (a triple negative breast cancer cell line), EC109 (an esophageal cancer cell line), ovcar3 (an ovarian cancer cell line) and BCPAP (a thyroid cancer cell line)(**Figure 4.2A**).

4.3.2 *ALK-I19* is present in a small amount compared to the fully spliced ALK (*FS-ALK*) transcripts

When we performed long-range RT-PCR using primer set #3 (from exon 1 to exon 29), we found only one band at ~4.3 kb, which is the approximate size of the *FS-ALK* transcript (i.e. 4,286 bp). Again, due to the deletions of exon 2 and 3, the amplicons from NB1 were slightly shorter than those from the other 3 cell lines. We speculated that the reason for not being able to detect *ALK-I19* with the long-range RT-PCR assay was because of its relatively low abundance, as compared to that of *FS-ALK*. In support of this concept, we performed quantitative RT-PCR (qRT-PCR) using primer set #4 (**Table 4.1**) covering a 99-bp segment in the mid-portion of I19 (**Figure 4.1B**),

and we found that *ALK-I19* was present at a very low level, with the cycle number being 32, as compared to 26 for all *ALK* detectable with primer #5 spanning from exon 24 to exon 25 (**Table 4.1**). A similar conclusion was obtained when we used primer set #6 and #7 (**Table 4.1**) simultaneously. Specifically, both primer sets shared the same 5' primer, being located in exon 18. In set #6, the 3' primer was located in I19, designed to detect *ALK-I19*. In set #7, the 3' primer was located in exon 20, designed to detect *FS-ALK* transcripts. As shown in **Figure 4.1C**, the *FS-ALK* transcripts (~289 bp) were substantially more abundant than *ALK-I19* (~172 bp).

4.3.3 *ALK-I19* is not derived from genomic DNA

We had considered the possibility that the *ALK-I19* was derived from genomic DNA, although several lines of evidence strongly argued against this possibility. First, as mentioned above, *ALK-I19* was detectable only in NB cell lines but not a number of other ALK-positive cell lines and normal tissues. As shown in **Figure 4.2A**, a number of ALK-negative cancer cell lines as well as reactive tonsils were also negative with the same RT-PCR assay. Second, we performed DNA sequencing of the entire amplicon generated from primer set #1 and #2, and we did not identify introns other than that of I19. Third, we did not observe any bands in the negative control lanes (**Figure 4.2**), in which reverse transcriptase was omitted.

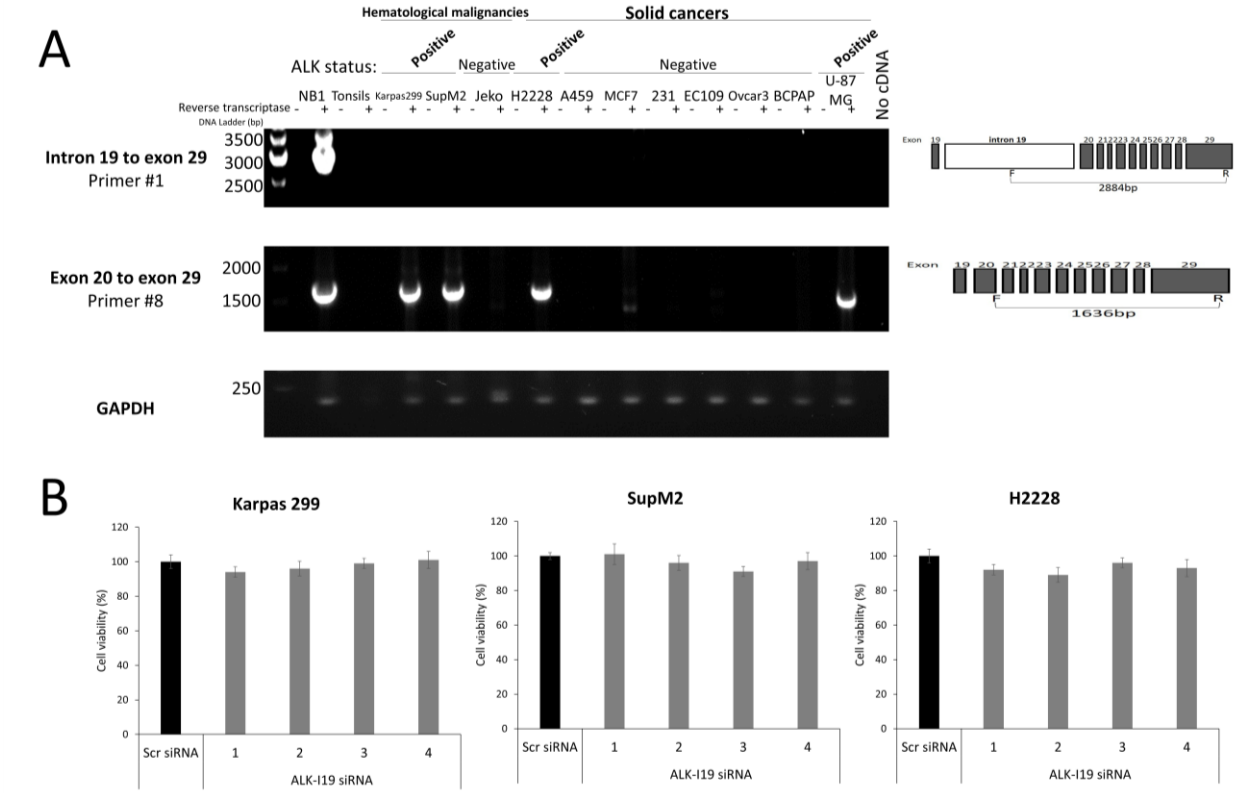


Figure 4.2 *ALK-I19* is not detectable in non-NB, ALK-expressing as well as ALK-negative human cell lines.

A. RT-PCR using primer set #1 was used to detect *ALK-I19*, and the result was positive for NB1 (positive control) and negative for reactive tonsils (negative control). Karpas 299 and SupM2 (ALK+ anaplastic large cell lymphoma cells expressing NPM-ALK), H2228 (a lung cancer cell line expressing EML4-ALK), U-87 MG (a glioblastoma cell line expressing wild-type full-length ALK), six ALK-negative cancer cell lines were all negative. The ALK-negative cell lines include Jeko (mantle cell lymphoma), A459 (lung adenocarcinoma), MCF7 (estrogen receptor-positive breast cancer), MB-MDA-231 (labeled as 231, triple-negative breast cancer), EC109 (esophageal squamous cell carcinoma), Ovcar3 (ovarian cancer) and BCPAP (thyroid cancer). **B.** siRNA knockdown of *ALK-I19* did not significantly affect the cell growth of Karpas 299, SupM2 and H2228. Cell growth was assessed using the MTS assay.

4.3.4 siRNA knockdown of *ALK-I19* dramatically reduces the total ALK expression

To assess the functional significance of *ALK-I19* in NB cells, we designed four intron 19-specific siRNA species, as illustrated in **Figure 4.3A**. The impact of these siRNAs on the expression levels of *ALK-I19* and *FS-ALK* was evaluated by using RT-PCR and two different primer sets, namely set #1 (to detect *ALK-I19*, ~2800 bp) and set #3 (to detect *FS-ALK*, ~4200 bp). As shown in **Figure 4.3B**, all 4 siRNA species were efficient in downregulating the expression level of *ALK-I19* in all 3 cell lines examined. Interestingly, all 4 siRNA species to knockdown *ALK-I19* were also efficient in downregulating the *FS-ALK*. Western blot studies also confirmed the dramatic reduction in ALK protein expression as a result of *ALK-I19* knockdown (**Figure 4.3C**).

4.3.5 siRNA knockdown of *ALK-I19* decreases cell growth and soft agar colony formation

We then assessed whether the siRNA knockdown of *ALK-I19* affects the growth of NB cells. As shown in **Figure 4.3D**, the growth of 3 NB cell lines, as determined by MTS assay for 48 hours, was significantly decreased when *ALK-I19* was knocked down by using siRNA ($p<0.01$), with the most dramatic effect observed in SK-N-SH cells. In contrast, in Karpas 299, Sup-M2 and H2228, all of which have no *ALK-I19*, siRNA knockdown of *ALK-I19* had no appreciable effect on the expression of *FS-ALK*, ALK protein expression and cell growth (**Figure 4.2B**). Next, we examined the effect of siRNA knockdown of *ALK-I19* on tumorigenicity. As shown in **Figure 4.3E**, siRNA knockdown of *ALK-I19* significantly downregulated the neurosphere formation ability (i.e. >50% reduction). These results support the biological importance of *ALK-I19* on regulating the total expression of ALK and thus, its oncogenic effects on cell growth and tumorigenicity.

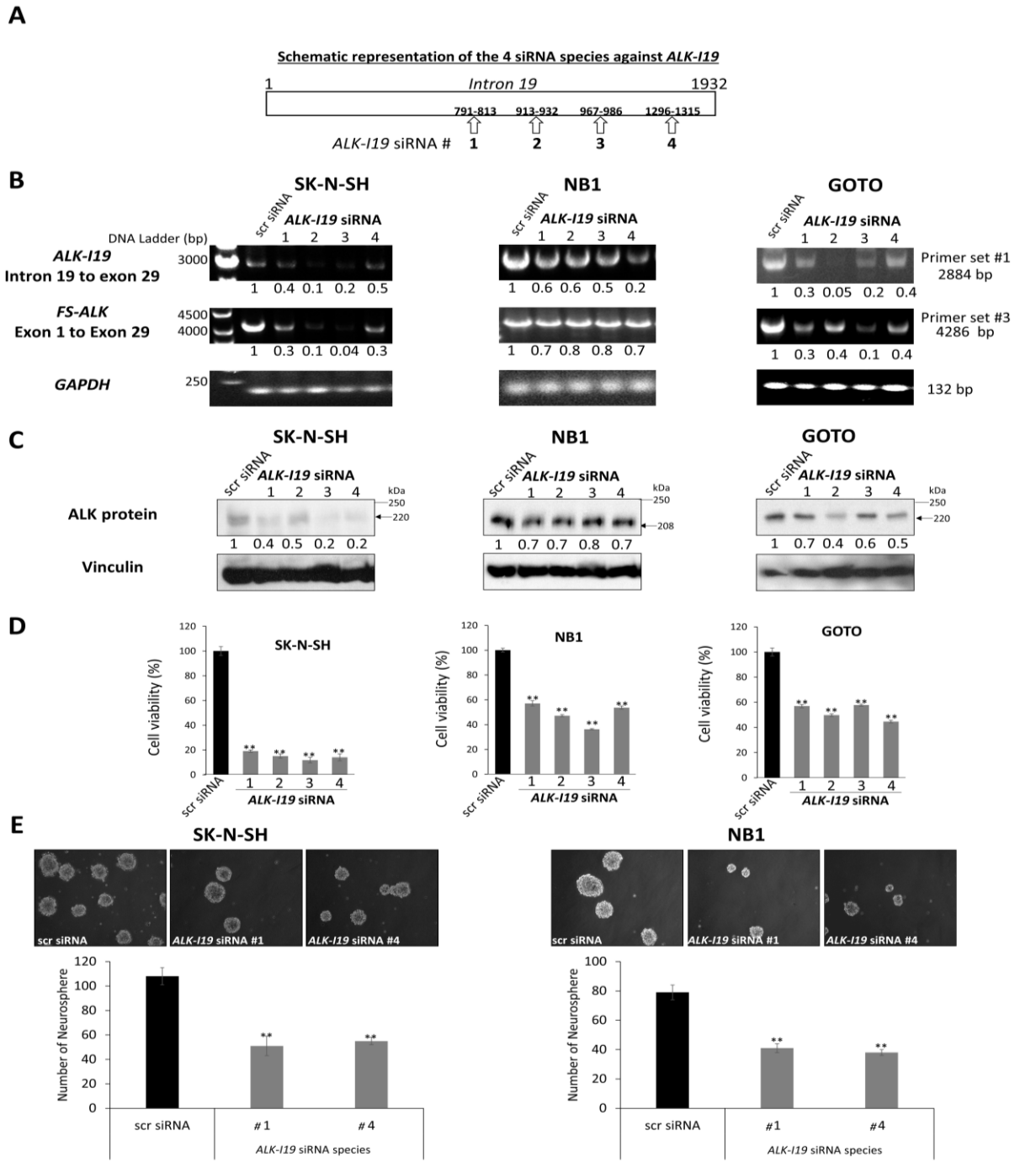


Figure 4.3 siRNA knockdown of *ALK-I19* decreases the expression of *FS-ALK*, ALK protein, cell growth and neurosphere formation in NB cells.

A. Schematic representation of the four *ALK-I19* siRNAs used. **B & C.** The expression levels of *ALK-I19* (primer set #1) and *FS-ALK* (primer set #3) as well as the ALK protein expression level

were reduced 48 hours following siRNA knockdown of *ALK-I19* in three NB cell lines. GAPDH was used as the loading control for the RT-PCR assay and vinculin was used as the loading control for the Western blots. The densitometry value of each band was normalized to that observed with scrambled (scr) siRNA. **D.** siRNA knockdown of *ALK-I19* significantly decreased cell growth at 72 hours, as assessed by MTS assay. Data is presented as mean ± standard deviation and statistical significance was determined using Student's *t* test. The *p* value for all runs is <0.01. Triplicate experiments were performed. **E.** siRNA knockdown of *ALK-I19* significantly decreased neurosphere formation, measured at 2 weeks after the siRNA knockdown. Data is presented as mean ± standard deviation and statistical significance was determined using Student's *t* test, n=3. The *p* value for all runs is <0.01. Triplicate experiments were performed.

4.3.6 *ALK-I19* is the precursor of the *FS-ALK* transcript

Based on our finding that siRNA knockdown of *ALK-I19* can efficiently down-regulate ALK expression, we hypothesized that *ALK-I19* is the precursor of *FS-ALK*. In support of this concept, we had collected additional evidence. First, we found that *ALK-I19* was confined to the nucleus in all 4 cell lines examined (**Figure 4.4A**); in contrast, *FS-ALK* was localized in both the nucleus and cytoplasm (**Figure 4.4B**). These results strongly suggest that *ALK-I19* is a non-coding transcript. *NBAT-1* (NB Associated Transcript 1), a long non-coding RNA that is exclusively localized in the nucleus (22), was used as a control for the efficiency of nuclear/cytoplasmic fractionation (**Figure 4.4C**). Second, when NB cells were treated with morpholino (an antisense oligomer) (28) that had been engineered to specifically block the splicing between exon 19 and intron 19, we found that the expression level of *FS-ALK* appreciably decreased at 48 hours, whereas the *ALK-I19* level remained largely unchanged (**Figure 4.4D**). Third, as shown in **Figure 4.4E**, cells treated with scrambled siRNA followed by actinomycin D for 24 hours expressed a substantial higher level of *FS-ALK* than cells treated with *ALK-I19* siRNA followed by actinomycin D. Actinomycin D is a transcription inhibitor that immobilizes DNA transcription initiation complex, thereby it prevents elongation of RNA chain (29).

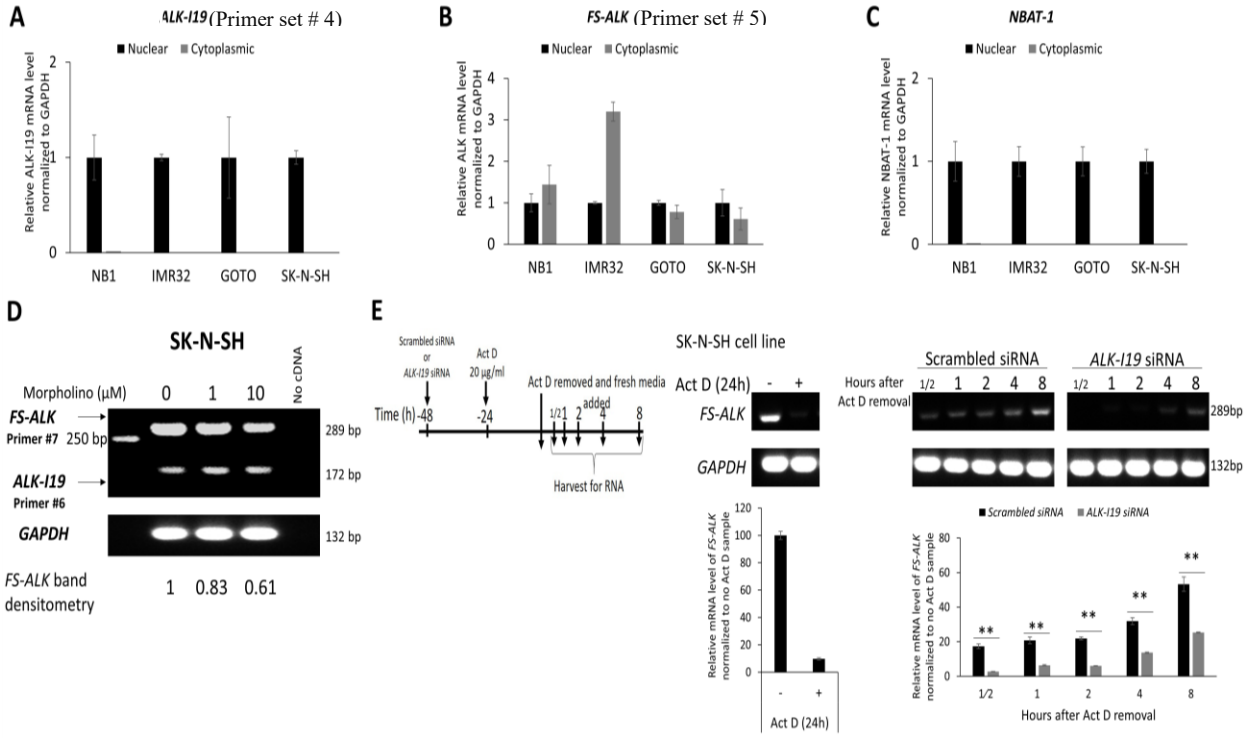


Figure 4.4 *ALK-I19* is the precursor of *FS-ALK*.

A-C. Measurement of *ALK-I19* in the nuclear and cytoplasmic fractions of NB cells showed that *ALK-I19* is expressed only the nucleus. In contrast, *FS-ALK* was found in both the nuclear and cytoplasmic fractions. *NBAT-1* was used as the control for the nuclear/cytoplasmic fractionation efficiency (30). **D.** Inhibition of the splicing between exon 19 and intron of *ALK* 19 using morpholino appreciably decreased the level of *FS-ALK* at 48 hours. The level of *ALK-I19* was not dramatically changed. **E.** SK-N-SH cells were first treated with either scrambled siRNA or *ALK-I19* siRNA for 24 hours. Subsequently, these cells were treated with 20 μg/ml Actinomycin D (Act D) for another 24 hours. After washing, cells were cultured in fresh growth media and RNA was harvested at different time points. This experiment was performed in the presence of either scrambled siRNA or *ALK-I19* siRNA. Cells treated with scrambled siRNA followed by actinomycin D expressed a substantial higher level of *FS-ALK* than cells treated with *ALK-I19* siRNA followed by actinomycin D. The densitometry value of each band was normalized to the cell sample receiving scrambled siRNA and no actinomycin D. Data is presented as mean ± standard deviation and the statistical significance was assessed using Student's *t* test, n=3.

4.3.7 Expression of the ALK-intron 19 variant in NB tumors

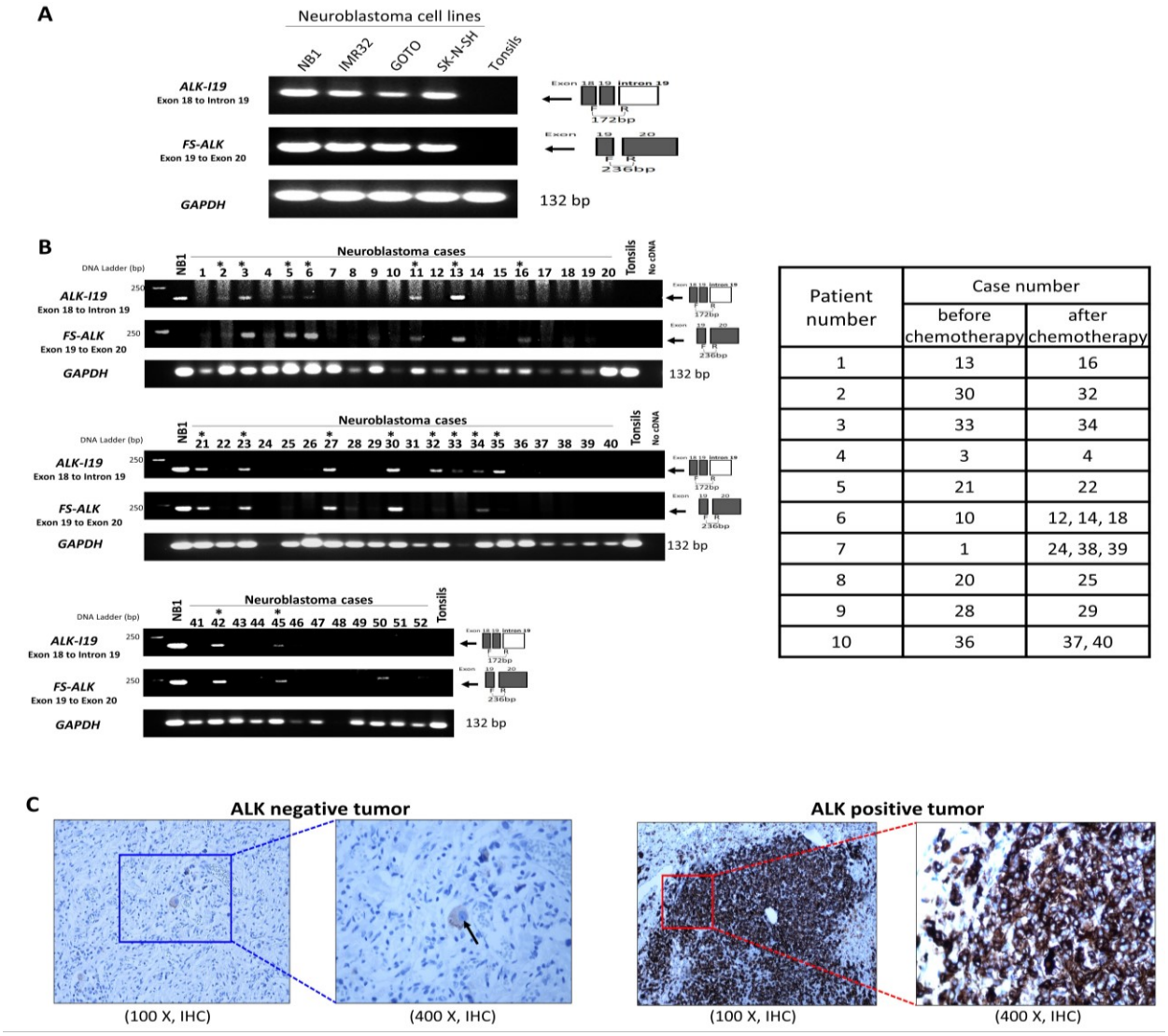
To prove that the expression of *ALK-I19* is not restricted to cell lines, we surveyed the expression of this variant in 52 formalin-fixed/paraffin-embedded NB tumors derived from 37 patients, for whom the demographic data is summarized in **Table 4.2**. To facilitate the detection of *ALK-I19* in paraffin-embedded tissues, we employed primer set #6 (exon 18 to intron 19, **Table 4.1**) with which a relatively short amplicon will be generated (i.e. expected size, 172 bp). Similarly, to detect *FS-ALK* in these tissues, we employed a new primer set (#9) with which a relatively short amplicon (i.e. expected size, 236 bp, exon 19 to exon 20, **Table 4.1**) can be generated. Of note, RT-PCR using primer set #9 was not expected to detect *ALK-I19*, since the expected product (i.e. >2 Kb) should not have survived the formalin-fixation.

As illustrated **Figure 4.5A**, RT-PCR using primer #6 yielded the expected *ALK-I19* product in all 4 NB cell lines but not reactive tonsils. RT-PCR using primer set #9 yielded the expected *FS-ALK* in all 4 NB cell lines. As illustrated in **Figure 4.5B**, 14 of 37 (38%) tumor samples obtained at diagnosis showed definitive evidence of *ALK-I19*. With the exception of three cases (#32, #33 and #35), these 14 positive cases also showed a readily detectable *FS-ALK* band. Of the remaining 23 tumors, *ALK-I19* was undetectable, and *FS-ALK* was not detectable in 20 cases, with tumor #4, #9 and #50 showing faint bands. The association between *ALK-I19* and *FS-ALK* is highly statistically significant (p=0.0001).

We then correlated these RT-PCR findings with IHC results, which are illustrated in **Figure 4.5C**. Thirteen out of the 14 tumors that expressed *ALK-I19* also showed strong ALK expression, as defined by >50% of tumor containing areas showing strong ALK immunostaining, a criterion used in a previous publication (24). Of the 23 tumors that were negative for *ALK-I19*, only one case was ALK-positive by IHC. Overall, we have identified a high statistical correlation between the presence of *ALK-I19* detectable by RT-PCR and ALK protein expression detectable by IHC (p<0.0001). Importantly, we observed that strong ALK staining intensity was largely restricted to the areas of undifferentiated histology, a finding that is also shared by another group (31). Accordingly, we found a significant correlation between the expression of *ALK-I19* and tumors with >50% undifferentiated histology (p=0.01). Of note, ganglion-like cells in the well-differentiated tumorous areas consistently expressed ALK weakly (**Figure 4.5C**). Thus, only the

intensely ALK-positive areas, typically found only in the areas of undifferentiated histology, were included for IHC scoring.

To determine whether the expression of *ALK-I19* is associated with other known features of NB, we correlated the expression of *ALK-I19* with a number of clinical and biological prognostic factors, as summarized in **Table 4.2**. Although *ALK-I19* did not significantly correlate with age, *ALK-I19* was more likely to be present in the older patients (i.e. >18 months, 64% versus 36%, p=0.10). *ALK-I19* was significantly associated with stage 4 diseases (p=0.04). Importantly, *ALK-I19* was significantly associated with the high-risk group based on Children's Oncology Group-risk classification (p=0.002) (32). *ALK-I19* was more common in cases with *MYCN* amplification but the correlation was not statistically significant (6/10 versus 8/27 cases, p=0.13).



Patient number	Case number	
	before chemotherapy	after chemotherapy
1	13	16
2	30	32
3	33	34
4	3	4
5	21	22
6	10	12, 14, 18
7	1	24, 38, 39
8	20	25
9	28	29
10	36	37, 40

Figure 4.5 The expression of *ALK-I19* and *FS-ALK* in formalin-fixed/paraffin-embedded (FFPE) NB tumors.

A. RT-PCR using primer set #6 (*ALK-I19*) and #9 (*FS-ALK*) was used to study 4 NB cell lines and a reactive tonsil. **B.** RT-PCR using primer set #6 and #9 was used to detect the expression of *ALK-I19* and *FS-ALK* in 52 FFPE tumors derived from 37 patients. Asterisks indicate cases that were labeled *ALK-I19*−positive, in which definitive *ALK-I19* bands could be identified. GAPDH was used as the loading control. The table on the right panel summarizes the 10 patients for whom pre- and post-chemotherapy tumor samples were obtained. Patient #1-5 had *ALK-I19*−positive tumor at diagnosis. Patient #1-3 had *ALK-I19*−positive tumors after chemotherapy whereas patient #4 and #5 had residual tumors that were *ALK-I19*−negative. Patient #6-10 had *ALK-I19*−negative tumors at diagnosis, and the residual tumors of all of these patients remained to be *ALK-I19*−negative. **C.** Immunohistochemical (IHC) detection of ALK showed heterogeneous staining

intensity in NB tumors. On the left panel in which an ALK-negative tumor is illustrated, ALK immunostaining was found only in the ganglion-like cells (black arrow) and the intensity was weak. On the right panel in which an ALK-positive tumor is illustrated, intense ALK immunostaining was found in large tumorous areas of undifferentiated histology.

Table 4.2 Clinicopathological characteristics of 37 neuroblastoma patients.

Clinical parameters	Number of patients (n=37)	*Positive ALK-I19 cases (n=14)*	*P* value	*Positive ALK by IHC (n=14)*	*P* value
Gender					
Male	24	8	*P*=0.49	8	*P*=0.49
Female	13	6		6	
Age at diagnosis					
≤ 18 months	20	5	*P*=0.10	6	*P*=0.33
> 18 months	17	9		8	
COG-risk groups					
Low-risk	9	0		1	
Intermediate-risk	11	3	*P*=0.002*	3	*P*=0.016*
High-risk	17	11		10	
INSS clinical stage					
I	3	0		1	
II	1	0		0	
IVS	4	1		0	
III	11	3	*P*=0.04*	3	*P*=0.04*
IV	18	10		10	
Primary tumor site					
Adrenal	23	11	*P*=0.17	11	*P*=0.17
Extra-adrenal	14	3		3	
Tumor Histology					
≥50% areas with undifferentiated histology	21	13		13	
<50% areas with undifferentiated histology	9	1	*P*<0.001*	1	*P*<0.001*
GNB	5	0		0	
GN	2	0		0	
MYCN					
Amplified	10	6	*P*=0.13	6	*P*=0.13
Not amplified	27	8		8	

Use Fisher's exact test for the *P* value (* statistically significant at <0.05). Children's Oncology Group-risk groups, COG-risk groups; INSS, International NB Staging System; NB, neuroblastoma; GNB, ganglioneuroblastoma; GN, ganglioneuroma.

In order to determine whether *ALK-I19* expression correlates with clinical outcome, we determined the overall survival (OS) and disease-free survival (DFS) data for our cohort (n=37). The median overall survival for this cohort was 33.1 months (range, 0 to 96 months, median follow-up 63 months). The 5 years OS and DFS for our cohort is shown in **Figure 4.6A** and **4.6B**. As expected, patients with *MYCN* amplification (n=10) survived significantly shorter compared to those with normal *MYCN* (n=27) (p=0.005) (**Figure 4.6C** and **4.6D**). Patients with *ALK-I19*—positive tumors (n=14) had inferior OS and DFS compared to those with *ALK-I19*—negative tumors (p=0.025 and 0.016, respectively) (**Figure 4.6E** and **4.6F**). Importantly, when we compared the clinical outcome of those with neither of these two abnormalities and those with both/either of these two abnormalities, we found a high statistical significance (p=0.002 for OS and p=0.001 for DFS) (**Figure 4.6G** and **4.6H**). Importantly, at the end of 5 years, all 19 patients with tumors carrying neither of these two markers survived, in contrast with only 10 of 18 patients with tumors carrying one or both of these two markers. Moreover, when we compared the clinical outcome of patients with *ALK-I19* and *MYCN* amplification, either one of these two aberrations, or neither, we found statistical significance indicating the poor outcome associated with both *ALK-I19* and *MYCN* amplification (p=0.012 for OS and p=0.011 for DFS) (**Figure 4.6I** and **4.6J**). Similar significance was observed when we performed this analysis using ALK expression detectable by IHC (**Figure 4.7**). In summary, the absence of both *ALK-I19* and *MYCN* amplification strongly correlated with an excellent clinical outcome in our cohort. Importantly, the inclusion of *ALK-I19* allowed the identification of a subset of NB patients with inferior outcome, despite the lack of *MYCN* amplification. In other words, *ALK-I19* appears to be a useful prognostic value, especially used in combination with *MYCN* amplification.

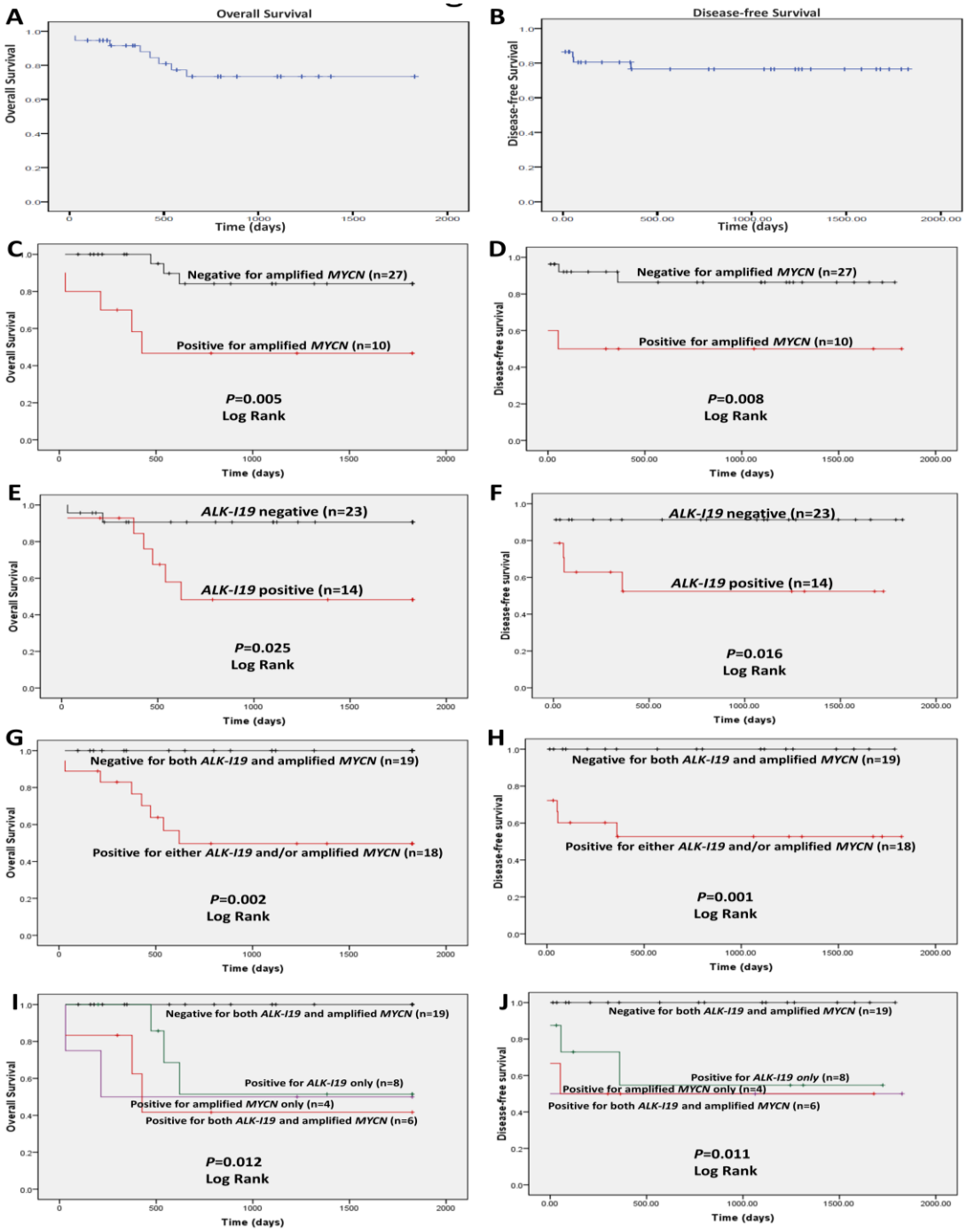


Figure 4.6 The prognostic significance of *ALK-I19* and *MYCN* amplification in 37 NB patients based on the overall survival (left panel) and the disease-free survival (right panel).

Kaplan–Meier survival is illustrated for the following: **A & B.** the entire cohort; **C & D.** patients with *MYCN* amplification versus those without; **E & F.** patients with *ALK-I19* versus those

without; **G & H.** patients with *ALK-I19* and/or *MYCN* amplification versus those negative for both genetic markers; **I & J.** patients with *ALK-I19* versus patients with *MYCN* amplification versus patients with both genetic markers versus those negative for both genetic markers.

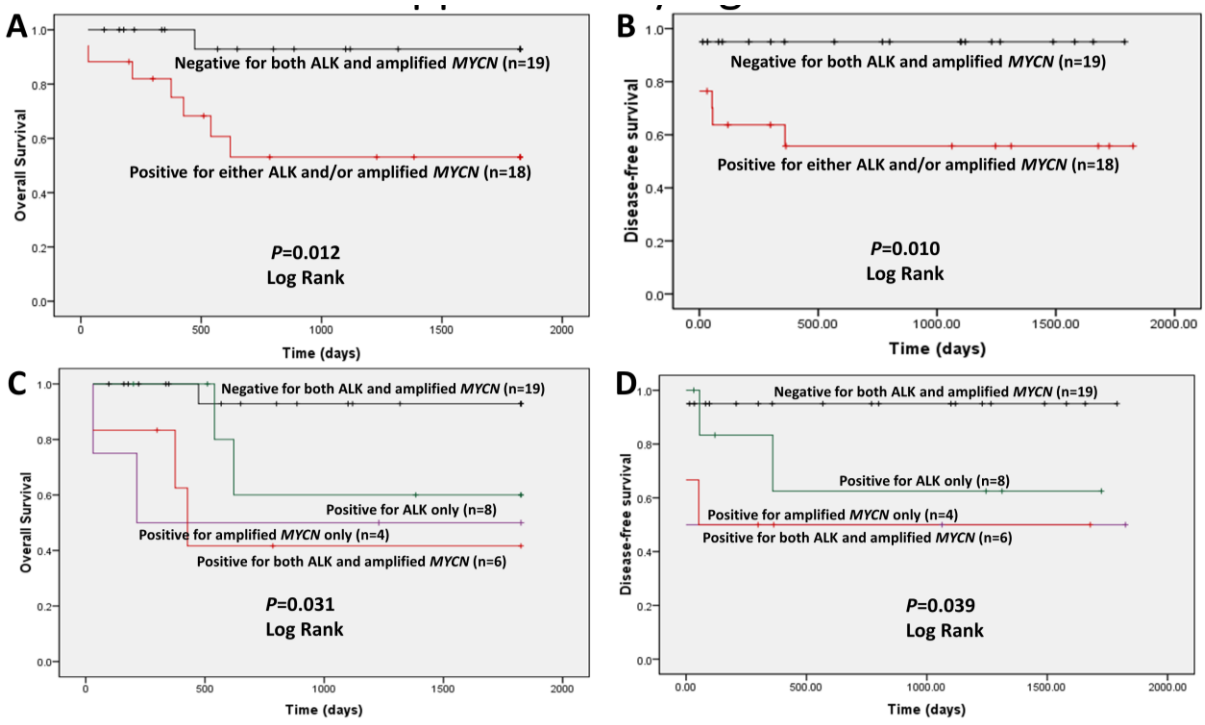


Figure 4.7 ALK expression detectable by immunohistochemistry is prognostically significant in NB patients based on the overall survival (left panel) and the disease free survival (right panel).

Kaplan–Meier survival is illustrated for the following: **A & B.** patients with ALK and/or *MYCN* amplification versus those negative for both markers; **C & D.** patients with ALK versus patients with *MYCN* amplification versus patients with both markers versus those negative for both markers.

Lastly, to determine whether *ALK-I19* expression changed following treatment, we identified 10 of 37 patients within our cohort who had sequential tumor samples after chemotherapy. Five of these 10 patients were positive for *ALK-I19*. Of these 5 patients, 3 remained to have *ALK-I19*—positive in the post-chemotherapy residual tumors. All of these 3 tumors were confirmed to be ALK-positive by IHC (i.e. >50% of the tumor), and the ALK-positive areas were largely restricted to the undifferentiated areas. For the remaining two patients whose tumors were *ALK-I19*—positive at diagnosis, the residual tumors became *ALK-I19*−negative, and ALK-negative by IHC and interestingly, most of the cells in these two post-chemotherapy tumor samples showed significant differentiation. The median overall survival was shorter for the patients whose tumors were *ALK-I19*—positive pre- and post-chemotherapy as compared to those whose tumors changed from positive to negative following chemotherapy (510 versus 1294 days). Of the five patients with *ALK-I19*—negative tumors at diagnosis, the post-chemotherapy residual tumors remained negative for *ALK-I19* expression and ALK expression by IHC. The median survival of the five *ALK-I19*—negative patients was 800 days.

4.4 Discussion

The key finding of this study is the identification of a novel *ALK* transcript, *ALK-I19*, which was detectable in 4/4 NB cell lines and in 38% of our cohort of NB tumors at time of diagnosis. *ALK-I19* is the precursor of *FS-ALK*, and abrogation of this transcript dramatically down-regulated *FS-ALK* as well as ALK protein expression. In NB tumors, we found that the expression of *ALK-I19* significantly correlates with >50% areas of undifferentiated histology, and a number of other known prognostic factors such as stage 4 disease, high-risk group and *MYCN* amplification. Importantly, all 19 patients whose tumors were negative for *ALK-I19* and/or *MYCN* amplification survived >5-years after the initial diagnosis. Based on our cell line studies, it appears that the expression of *ALK-I19* is not dependent on the mutational status of ALK, since it was identified in 4 NB cell lines that carry wild-type *ALK*, amplified *ALK* or mutated *ALK*.

To our knowledge, the inclusion of I19-containing *ALK* transcripts has been reported in 6 ALK-expressing tumors described in five studies published between 2008-2013 (17-21). Of these 6

cases, 5 were non-small cell lung cancer expressing *EML4-ALK* and one case was myofibroblastoma expressing *PPFIBP1-ALK*. Sequencing data is available for these 6 cases; all include a portion of the 3' end of I19 ranging from 12 bp to 117 bp. In all 6 cases, the 5' end of the included I19 directly connects with the fusion gene partners (i.e. *EML4* or *PPFIBP1*) and 3' end of the included I19 connects with exon 20 of *ALK*. In 5 cases, RT-PCR studies revealed that these I19-containing *ALK* transcripts are the only *ALK* mRNA species detectable; in the remaining one case, both I19-containing and the typical *ALK* fusion transcripts without any introns were identified. In none of these studies, the ALK protein was fully analyzed and whether the I19 was represented at the protein level is unknown. The significance of the inclusion of I19 in these *ALK* transcripts is also largely unknown. In a more recent study of melanomas published in 2015, *Wiesner et al* reported the existence of I19-containing *ALK* transcripts in which only the 3' end of I19, approximately 400 bp in length, represents the beginning of the *ALK* transcript and producing a novel initiation transcription site; these aberrant *ALK* transcripts were found in 11% of their cohort of melanomas (21). Importantly, these transcripts and the resulting alternative initiation site lead to the production of truncated ALK proteins (ALK ^{ATI}) that are oncogenic (22). Interestingly, while portions of I19 appear to have the propensity of being included in the *ALK* transcripts expressed in some cancer cells, we are not aware of any example in which the inclusion of other *ALK* introns is described. In this regard, we speculate that the exclusive retention of the full I19 in this setting may be linked to the unique biochemical characteristics of this specific intron. In support of this concept, almost all *ALK* translocations in ALK-expressing human cancers have breakpoints located at the junction between intron 19 and exon 20 (33).

ALK-I19 is unique in several aspects. In contrast with these I19-containing *ALK* transcripts described above, our identified *ALK-I19* contains the entire I19 that is ~2 kb. Secondly, in contrast with the I19-containing *ALK* transcripts reported previously, which appeared to be the only *ALK* transcript expressed in the cancer cells, *ALK-I19* was the minor *ALK* mRNA species, being over-shallowed by the abundant *FS-ALK* in NB cells. Third, *ALK-I19* is a precursor of *FS-ALK*, and we had no evidence that the intron 19 was translated into proteins detectable by Western blots using two different anti-ALK antibodies. In contrast, in the previous reports of the I19-containing transcripts, it is highly likely the portions of I19 were translated, since these I19-containing transcripts were the only *ALK* transcripts detectable. Lastly, the two I19-containing ALK

transcripts were found in the two fusion ALK genes (namely *EML4-ALK* and *PPFIBP1-ALK*); in contrast, *ALK-I19* was found to be expressed in NB cells expressing *ALK* without fusion partners.

Our data suggests that *ALK-I19* is unique to NB cells. Specifically, it was not found in normal tissues nor other ALK-expressing human cancer cells. Furthermore, this aberrant transcript was not detectable in the RNA samples derived from U-87 MG cells, a glioblastoma cell line that was derived from malignant astrocytes (34). Taken together, it is possible that *ALK-I19* represents a relatively specific biological marker of undifferentiated NB cells. In other words, NB tumors expressing a relatively high proportion (i.e. 50%, as shown in our study) of areas of undifferentiated histology will express *ALK-I19* detectable by using our RT-PCR assay, whereas tumors in which the areas of undifferentiated histology represent only a minor component will have no detectable *ALK-I19*. It is of interest to test this hypothesis by including more NB tumors and other forms of ALK-expressing human cancer cells in the future studies.

The retention of specific introns or portions of introns has been described in normal cells in plants and animals (35). The expression of intronic mRNA species is particularly high in the brain, especially the fetal brain (36). The biological significance of these unusual transcripts has been examined, but it is not completely understood. Nonetheless, in a number of scenarios, intron-retained transcripts serve as reservoirs, which can boost protein production upon specific stimuli (37, 38). For example, one study has identified an unusual intron 3-containing *ApoE* mRNA species that is detectable in cortical and hippocampal neuronal cells (34). It was suggested that the expression of this specific transcript contributes to the relatively rapid upregulation of the ApoE protein in response to cellular stress (34). In other words, intron 3-retaining *ApoE* is believed to provide a '*ApoE* mRNA reserve pool', which can be called upon to synthesize a large of ApoE protein within a relatively short timeframe. Whether the existence of intron-retaining mRNA species in cancer cells provides the same purpose requires further investigations.

Intron-retaining transcripts also have been described in cancer cells, although their significance has not been extensively studied (37). In a review paper by *Wong et al* (37), RNA sequencing has identified intron retention in thousands of genes expressed in tumors of lung, breast, kidney, prostate and head/neck; the biological functions of these transcripts are largely unknown. A few studies have provided specific examples. *RET*, which encodes another tyrosine kinase, can be

expressed as an intron 2-retaining variant that is detectable in 19% of pheochromocytomas (39). Intron 4-retaining *CCDN1* expressed in prostate and esophageal cancers was found to translate into a truncated cyclin D1 protein which has oncogenic effects (40, 41). With respect to the role of entire I19 retention (*ALK-I19*), we speculate that the '*ApoE* model' may be more applicable. In our model, *ALK-I19*, the precursor of *FS-ALK*, is stored in the nuclei of NB cells, possibly predominantly in those that are undifferentiated. In response to adverse events, such as hypoxia or exposure to chemotherapeutic agents, cell survival signals may be induced including the ability to upregulate the expression of ALK, a protein known to promote survival in cancer cells (3). This may provide one of the biochemical explanations to the prognostic significance of *ALK-I19* in NB.

Our finding that ALK expression detectable by IHC correlates with prognosis is in keeping with the conclusions of 4 previously published studies (6, 7, 24, 31). It is important to point out that, despite the fact that different anti-ALK antibodies were employed in each of these 4 published studies, and that the scoring criteria used in these studies were not uniform, the prognostic significance of ALK immunostaining scores was identified in these studies. However, all four studies used a cut-off of 50% ALK-positive immunostained tumor cells, which formed the basis of the scoring criteria used in our current study. In our experience, the staining intensity, in addition to the percentage of tumor cells showing ALK immunostaining, is important. In fact, 3 of these 4 published papers incorporated the staining intensity in their scoring. The rationale is related to the fact that the ganglion-like cells in the differentiated areas are consistently weakly ALK-positive. By including the most intensely stained areas only, one can essentially exclude the well-differentiated areas and take into the account the poorly differentiated areas exclusively, which are strongly ALK positive by IHC and likely express the *ALK-I19*. With these considerations, it is not surprising that we have identified a strong correlation between the expression of *ALK-I19* and the proportion of poorly differentiated areas, as well as the frequency of observing >50% tumor areas showing strong ALK immunostaining.

One of the most important findings of this study is related to the potential clinical utility of *ALK-I19* expression in primary NB tumors. The expression of this aberrant *ALK* transcript, being found approximately one-third of the NB patients, significantly correlated with a shorter survival. Our analyses have also shown that *ALK-I19* may have additional prognostic power when used in

combination with *MYCN* amplification. Specifically, in the absence of both *ALK-I19* and *MYCN* amplification, all 19 (100%) patients survived and 10 of these 19 (55%) patients were disease-free at 5 years. In comparison, only 10 of 18 patients with either *ALK-I19* or *MYCN* amplification survived at 5 years (56%) and only 4 of these 18 patients (22%) have no evidence of disease at 5 years. Perhaps the most interesting subset of patients are those with tumors that lacked *MYCN* amplification but were positive for *ALK-I19* expression (n=8). The survival of this subset of patients was found to be comparable to those with tumors harboring *MYCN* amplification alone. Based on these findings, we conclude that the detection of *ALK-I19* can substantially improve the prognostic value of *MYCN*, and we propose that these findings to be further validated in a larger cohort. Although *ALK-I19* was found to correlate with ALK expression by IHC, we believe that detection of *ALK-I19* by RT-PCR is more advantageous than detection of ALK by IHC, since the detection of *ALK-I19* is less subjective and prone to the variability related to the use of the IHC methods and antibodies employed. Furthermore, this assay can be performed on routine-fixed/paraffin-embedded tissues, and this facilitates its adoption in routine pathology laboratories.

In conclusion, we have identified *ALK-I19*, a novel *ALK* transcript expressed exclusively in a subset of NB. *ALK-I19* is the precursor of *FS-ALK*, and our data suggests that it is likely a biological marker of undifferentiated NB cells. Furthermore, the prognostic significance in our small cohort suggests that the status of *ALK-I19* may be used together with *MYCN* amplification to further identify groups of patients with particularly poor outcome and may be useful in risk stratification. Further studies will be required to determine whether the *ALK-I19* expression has any impact on response to ALK inhibitors or other therapies.

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Chapter 5

General Discussion and Conclusions

5.1 Crizotinib sensitivity in ALK$^+$ cancer cells is dictated by the structure of ALK and some of its binding partners

5.1.1 Major challenges in overcoming crizotinib/TKI resistance in ALK$^+$ cancer patients

Crizotinib represents a breakthrough treatment for some ALK$^+$ NSCLC patients and there is an increased urgency for it to be approved for other ALK$^+$ cancer patients (1, 2). However, there is a wide range of responsiveness to crizotinib treatment among different ALK$^+$ cancer types with largely unknown mechanisms (3, 4). Specifically, resistance to crizotinib invariably develops mostly within a year (5) and the current approach to overcome crizotinib resistance mainly relies on second and third generation tyrosine kinase inhibitors (TKIs), with over 11 inhibitors being developed (6, 7).

With the increased experience about TKI resistance, the clinical response to the next-generation TKIs is commonly highly variable and unpredictable (7). For instance, third-generation EGFR TKIs are being developed as part of the strategy to overcome treatment resistance to first- and second-generation EGFR TKIs in lung cancer patients (8), however, resistance to third-generation EGFT TKIs, such as AZD9291 and HM61713, also developed (9). A pervading theme regarding resistance to TKI therapy is its mediation by secondary mutations, which has not been resolved through introducing new generations of more TKIs. For example, despite the clinical efficacy of the first-, second- and third-generation BCR–ABL inhibitors, resistance occurs invariably and more than 50 distinct point mutations encoding single amino-acid substitutions in the kinase domain of the *BCR–ABL1* gene have been detected in patients with imatinib-resistant chronic myeloid leukemia (CML) (10, 11). Adding more inhibitors is not working, as shown for other tyrosine kinase inhibitors. Therefore, it is crucial to identify new strategies to overcome the TKI resistance in ALK$^+$ cancer patients.

5.1.2 A novel mechanism underlying crizotinib/TKI resistance in ALK$^+$ cancers

In chapter 2 of this thesis, I have provided a novel understanding of crizotinib resistance by proving that the differential sensitivity to crizotinib in ALK-positive cancers is dependent on the drug

binding ability. I also demonstrated that the crizotinib—ALK binding is largely determined by the biochemistry/functional status of ALK. Interestingly, my studies identified a novel role of an ALK-interacting protein, β-catenin, in blocking the crizotinib—ALK binding and, thereby, mediating resistance to crizotinib treatment. I believe my current work has highlighted the importance of protein-protein interactions in challenging cancer resistance phenotypes.

I have employed CETSA, a recently described method that allows rapid and simple assessment of target engagement of drugs in a cellular context (12-14), as a tool to evaluate the physical binding of crizotinib to ALK in intact cells. The current read-out used to measure the effect of ALK inhibitors focused on phenotypic assays in which the response to an inhibitor is based on a functional readout, such as changes in the phosphorylation status of downstream targets or impact on cellular viability (15). Therefore, the use of CETSA is advantageous in that the drug-target interactions are evaluated in a relevant cellular context. My work identified a significant positive correlation between crizotinib—ALK binding and the observed IC_{50}, which provides a logical justification for the differential responsiveness. Additionally, it proves that the CETSA assay is a very useful tool to predict the crizotinib sensitivity in different ALK-carrying cancer types.

To conclude, my studies have provided direct evidence that crizotinib—ALK binding is the key determinant and predictor of crizotinib sensitivity in ALK^+ cancer cells. Furthermore, the finding that β-catenin as an ALK-binding protein can substantially contribute to crizotinib resistance has opened a new avenue in overcoming the clinical resistance to ALK inhibitors.

5.2 Intra-tumoral heterogeneity dictates crizotinib resistant phenotype in neuroblastoma

One of the key findings in Chapter 3 is related to the identification of two phenotypically distinct cell populations in NB cells based on their differential responsiveness to the Sox2 reporter. Compared to reporter unresponsive (RU) cells, reporter responsive (RR) cells exhibited significantly higher tumorigenicity, neurosphere formation, chemoresistance, and expression of CD133 and nestin, two markers previously shown to be associated with cancer stemness in NB (16, 17). Moreover, I found that RR cells are significantly more resistant to crizotinib than RU

cells. Dr. Lai's lab has previously identified similar intra-tumoral dichotomy in other types of cancer using the SRR2 reporter. Specifically, we have reported such dichotomy in ER^+ breast cancer, TNBC, ESCC and ALK^+ALCL (18-21). Thus, it appears that the RU/RR dichotomy and their difference in tumorigenic features may be generalized to many types of human cancers.

While the SRR2 reporter contains a naturally occurring Sox2 binding consensus sequence present in the enhancer of hundreds of genes in the genome, it proved to represent a useful surrogate marker for the CSCs/RR phenotype and its associated cancer stemness features in multiple cancer types. We now know that while SRR2 is highly specific to Sox2 in some cancer types such as ER^+ breast cancer, we found that SRR2 may be potentially recognized by other stemness-associated transcriptional factors including MYC, STAT3 and NFκB based on the sequence analysis of SRR2 (19, 22). More specifically, I have actively participated with Dr. Lai's lab members in a few recently published studies that showed the SRR2 reporter is regulated by Sox2 and MYC in ALK^+ALCL and by MYC but not Sox2 in TNBC and ESCC (19, 22, 23). Importantly, the more tumorigenic and stemness features in RR cells were reproducible despite the different SRR2 driver. Thus, the SRR2 reporter represents a transcriptional signature of this small subset, which allowed prospective identification of CSCs.

While acquired resistance to crizotinib (which is caused by post-treatment changes such as alteration in drug targets and the activation of compensatory survival signaling pathways) is under extensive investigations (24), intrinsic resistance (which includes the factors that exist before treatment such as the presence of CSCs) is almost lacking in ALK^+ cancers. In chapter 3, my studies demonstrated, for the first time, that β-catenin plays a direct role in conferring the intrinsic resistance to crizotinib in the more tumorigenic NB cell population (i.e. RR cells). I believe that the results from this chapter reinforce the concept that high expression of β-catenin could be responsible for the crizotinib resistance.

To conclude, this study has highlighted intra-tumoral heterogeneity using a study model that is based on the differential responsiveness to an SRR2 reporter. Furthermore, the finding that β-catenin expression level is enriched in the more tumorigenic cells (i.e. CSCs/RR cells) can substantially contribute to crizotinib resistance and has opened a new avenue for further investigation for the role of CSCs in the clinical resistance to tyrosine kinase inhibitors.

5.3 *ALK-intron 19* (*ALK-I19*) is a novel *ALK* transcript expressed in neuroblastoma

Aberrant expression and activation of ALK has been implicated in various human cancers through either generation of fusion proteins, gene amplification or single nucleotide mutations (24). Specifically, there are various forms of the full-length ALK protein that are expressed in NB including wild-type, amplified and different mutants (25). While data about the pattern of ALK expression is almost restricted in analyzing the *ALK* gene (i.e. DNA), analyzing *ALK* mRNA for additional *ALK* transcript(s) is lacking. In chapter 4, I have identified a novel *ALK* transcript that retains intron 19. This *ALK-I19* mRNA was detected in 4 out of 4 NB cell lines while being undetected in other ALK^+ or ALK^- cancer cells. This work provides the first evidence that an additional form of *ALK* mRNA transcript exists in an ALK-expressing cancer type, NB, through intron retention.

It is not unreasonable to speculate that multiple *ALK* mRNA transcripts could exist. This speculation is based on two main reasons. First, the leukocyte tyrosine kinase (LTK), a tyrosine kinase that shares 71% sequence homology with ALK, demonstrates extensive alternative splicing that gives rise to five LTK isoforms (26), although none of the LTK transcripts had intron-retention. Second, *Morris et al* screened cDNA libraries of two cell lines derived from the skeletal muscle tumor rhabdomyosarcoma (RMS17-2 and Rh30) using a probe corresponding to 3'-ALK-specific sequences and identified two *ALK* transcripts of 6.5 kb and 8.0 kb (27). In their subsequent work, they characterize the 6.5 kb *ALK*, which turns out to be exactly 6226 bp, but they did not characterize the 8.0 kb because, as they mention, it was significantly less abundant than the 6.5 kb transcript (28). While the authors suggest that a second *ALK* transcript represents differentially spliced *ALK* mRNA, no further studies has been performed. My results re-emphasize the importance of analyzing the existence of additional *ALK* transcript(s) in normal tissues as well as in cancer cells.

My results showed that *ALK-I19* could be a very long transcript (> 6 kb) that is likely a precursor for the fully spliced *ALK* transcript. It may be challenging to characterizes the entire *ALK-I19* transcript since the current cloning and sequencing approaches are unable to characterize some well-documented long noncoding RNAs (>5 Kb) such as *Xist* and *Air* (29). In agreement with our

observation that *ALK-I19* transcript is not detected in fetal brain, a previous study sequenced total RNA from fetal brain and found a large fraction of reads (up to 40%) within introns, however, ALK was not among these reported genes (30). On the other hand, my results also suggest that NB cells express *ALK-I19* transcript regardless of the amplification/mutation status. Specifically, the 4 examined NB cell lines in this study carried different *ALK* status, however, *ALK-I19* was expressed in all of them.

5.4 *ALK-I19* is expressed in a subset of neuroblastoma patients and may have additional prognostic power when used in combination with *MYCN* amplification

One of the most important findings of my studies in chapter 4 is the detection of *ALK-I19* expression in one-third of the NB patients and its potential clinical utility. Specifically, my study showed that *ALK-I19* expression significantly correlated with a shorter survival. This result suggests that *ALK-I19* may have additional prognostic power when used in combination with *MYCN* amplification. Importantly, in the absence of both *ALK-I19* and *MYCN* amplification, all analyzed patients (n=19) survived and half of them were disease-free at 5 years. In comparison, only 10 of 18 patients with either *ALK-I19* or *MYCN* amplification survived at 5 years (56%) and only 4 of these 18 patients (22%) have no evidence of disease at 5 years.

The most interesting subset of patients in this study are those with tumors that lacked *MYCN* amplification but were positive for *ALK-I19* expression (n=8). Interestingly, the survival of this subset of patients was found to be comparable to those with tumors harboring *MYCN* amplification alone. Based on these findings, my study concludes that the detection of *ALK-I19* can substantially improve the prognostic value of *MYCN*.

To conclude, this study reports a novel *ALK* transcript, *ALK-I19*, which was detectable in a subset of NB tumors. *ALK-I19* was not found in normal tissues, non-NB/ALK-expressing cells and non-NB/ALK-negative cells. Thus, *ALK-I19* has the potential of serving as a diagnostic marker. The functional significance of *ALK-I19* was determined as it appears to be a precursor for the fully spliced *ALK*. Importantly, *ALK-I19* was found to be highly prognostically useful in NB patients. Specifically, patients with NB tumors carrying *ALK-I19* but no evidence of *MYCN* amplification

performed as poorly as those with *MYCN* amplification. My data suggests that the status of *ALK-I19* may be used together with *MYCN* amplification to further identify groups of NB patients with particularly poor outcomes.

5.5 Study limitations

My study mainly relied on the use of cell lines, however, I believe this is unavoidable in order to acquire enough cells required to study crizotinib resistance and cell signaling in a cost-effective and efficient manner. Dr. Lai's lab chose our cell lines carefully based on their genetic, epigenetic, phenotypic and clinical profiling. Furthermore, all cell lines used in my studies have been authenticated using short tandem repeat DNA profiling (from TCAG Genetic Analysis Facility, Toronto, CA). In fact, the results of the *in vitro* studies using ALK^+ cell lines, in which they display a wide range of crizotinib sensitivity correlates well with the clinical observation.

Another limitation of my studies in Chapter 3 lies profoundly on the simplified concept that there are two subsets in the neuroblastoma cells. Neuroblastoma and other cancers are a collection of sub-clones based on their survival abilities in a changing, multi-component microenvironment. During the purification of RU cells and RR cells, I observed as a spectrum of GFP expression which might represent heterogeneity of cancer cells. My choice to narrow to two subsets serves to simplify and focus the concept of tumor cell heterogeneity.

In Chapter 4, I found that the level of *ALK-I19* is of low abundance in the 4 analyzed cell lines compared to the (*fully-spliced*) *FS-ALK*. However, this seems not the case when I performed the analysis using patient samples. This could be due to the limitation of using cell lines which could carry a very active splicing machinery that quickly gave rise to *FS-ALK*. For instance, *Waks et al.,* showed that the tight control of the regulatory splicing machinery is compromised in HeLa cells (31). Therefore, it might be more informative to study *ALK-I19* regulation using patient-derived cell lines.

5.6 Future directions

Since I used only 7 ALK^+ cell lines and found a significant positive correlation between crizotinib—ALK binding and the observed IC_{50}, it is worthwhile to expand the analysis to include more ALK^+ cell lines as well as patient-derived cell lines. Furthermore, my results from Chapter 2 and Chapter 3 suggest CETSA as a very useful tool in predicting crizotinib sensitivity. It is worthwhile investigating whether CETSA can be used in the clinical setting to predict drug sensitivity in large-scale studies employing clinical samples.

My data in Chapter 2 and Chapter 3 suggests that other ALK-interacting proteins (yet to be identified) may continue to hinder the binding of crizotinib to ALK, even in the absence of β-catenin. Therefore, it is logical to identify other ALK-interacting proteins that correlate with elevated crizotinib IC_{50}. First, we should define the crizotinib IC_{50} in a panel of *ALK*-expressing plasmids that carry different mutations, including known secondary mutations such as 1151Tins, L1152R, C1156Y, F1174L, L1196M, G1202R, G1206Y and G1269A. Mutations that will cause an increase in the crizotinib IC_{50} will be called resistant forms, while mutations that will not cause an increase in the crizotinib IC_{50} will be called sensitive forms. Second, we should identify ALK-interacting proteins that are associated with each mutated ALK using mass-spectrometry. Proteins that are shared between resistant and sensitive forms will be excluded. For example, we expect to observe HSP proteins to be bound by both resistant and sensitive forms. Third, we will need to do target validation by performing siRNA knockdown for each ALK-interacting protein and perform CETSA as well as cell viability assays to confirm which ALK-interacting protein is the right candidate. Finally, we will need to design small molecule inhibitors that can disrupt the interaction between ALK and its interacting protein.

My data suggests that the interaction between oncogenic tyrosine kinases and various cellular signaling proteins may be more complex than previously believed. For instance, I found that β-catenin contributes, through its interaction with ALK, to the differential crizotinib sensitivity between different ALK^+ cell lines and between RU and RR cells that derived from the same cell line. Further studies to investigate this level of regulation may be warranted.

Many approaches were suggested to treat ALK^+ cancers through interfering with ALK processing, such as targeting its folding, or ALK phosphorylation using ALK inhibitors (24). Examples for the first approach (i.e. targeting ALK processing) include targeting the ALK chaperone protein HSP90 (32, 33) and the inhibition of N-linked glycosylation of ALK (34). However, this approach completely lacks specificity against ALK or against cancer cells as both processes are crucial for non-oncogenic proteins as well as for normal cells. For the second approach, there are many challenges to overcome differential responsiveness and secondary resistance for the current ALK inhibitors, especially for NB patients who carry different ALK aberrations. Therefore, an alternative approach is urgently needed. In recent years, several reports have highlighted that splicing patterns are frequently altered in cancer, and mutations in genes encoding spliceosomal proteins as well as mutations affecting the splicing of key cancer-associated genes are enriched in cancer (35). Additionally, many spliceosome inhibitors are currently under extensive investigation (36). Thus, it is warranted to look into ALK splicing mechanisms and delineate its splicing regulatory networks in NB cells.

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