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ABSTRACT

Biopsies and cell lines of NK/T-cell lymphoma, nasal-type (NKTCL) were subject to combined gene expression profiling and array-based comparative genomic hybridization analyses. Compared to PTCL, NOS, NKTCL had higher transcript levels for NK-cell markers and cytotoxic molecules, especially granzyme H, a novel sensitive biomarker of NKTCL. Compared to normal NK cells, tumors were closer to activated than resting cells and overexpressed several genes related to vascular biology, EBV-induced genes and *PDGFRA.* Notably, PDGFRα and its phosphorylated form were confirmed at the protein level, and *in vitro* the MEC04 NKTCL-cell line was sensitive to imatinib. Deregulation of the AKT, JAK-STAT and NF-κB pathways suggested by bioinformatical analysis, was corroborated by nuclear expression of phosphorylated AKT, STAT3 and RelA in NKTCL, and several deregulated genes in these pathways mapped to regions of recurrent copy number aberrations (*AKT3* (1q44), *IL6R* (1q21.3), *CCL2* (17q12), *TNFRSF21* (6p12.3)). Several features of NKTCL uncovered by this analysis (overexpression of VEGFA and its receptor KDR by the tumor cells, overexpression of *MET-HGF*) suggest perturbation of angiogenic pathways. Integrative analysis also evidenced deregulation of the tumor suppressor *HACE1* in the frequently deleted 6q21 region. This study highlights emerging oncogenic pathways in NKTCL and identifies novel diagnostic and therapeutic targets.

INTRODUCTION

Several NK/T-cell lymphoma entities have a predilection for extranodal locations. One of the most common of these overall rare lymphoma entities is NK/T cell lymphoma, nasal type (NKTCL). The disease, most prevalent in Asian, Central and South American populations, most commonly arises in the nasal cavity or adjacent structures, but can also occur in other extranodal sites.[1] This lymphoma exhibits an angiocentric and angiodestructive growth pattern characteristically associated with necrosis and ulceration. The tumor cells typically express CD2, cytoplasmic CD3 (CD3ε chain) and CD56, are negative for CD5, CD4 and CD8 and have a cytotoxic immunophenotype with expression of perforin, granzyme B (gzm B), and T cell-restricted intracellular antigen (TiA1). Most cases derive from NK cells and lack T-cell receptor (TCR) gene rearrangement while a small proportion of cases have the phenotype and genotype of cytotoxic γδ or αβ T cells. Accordingly, the expression of killer immunoglobulin-like receptors (KIRs) has been documented in these tumors.[2]

In virtually all cases, most neoplastic cells harbor clonal episomal Epstein-Barr virus (EBV), suggesting the implication of the virus in tumor pathogenesis. The genetic alterations of NKTCL reported in a few studies include gain on chromosome 2q, losses of chromosomes 6q and 1p, and occasional presence of isochromosome 7q.[3-6]

Even with intensive therapies combining multi-agent chemotherapy and involved field radiotherapy, the prognosis of NKTCL remains poor with 5-year overall survival ranging from 42% to 64% in four recent studies,[7-10] and novel alternative approaches are needed. Several factors have been incriminated to account for aggressiveness or poor outcome, including Fas and p53 mutations,[11,12] expression of the multidrug resistance (MDR) 1 gene product P-glycoprotein,[13] absence of CD94 transcript[14] and gzm B inhibitor PI9.[9] However, the mechanisms involved in resistance to therapy remain poorly understood.

Genome-wide profiling studies of NKTCL are scarse,[3,15] likely reflecting not only the rarity of the disease, but also quantitative and qualitative restrictions imposed by small and often necrotic diagnostic samples. In one recent study, Iqbal et al. focused on in-depth genomic and transcriptomic analysis of specific chromosomal regions, which led them to identify candidate suppressor genes mapping to 6q.[5] In the current study, we analyzed a series of NKTCL samples in relation to normal cells and peripheral T-cell lymphoma, not otherwise specified (PTCL, NOS), with the aims to (1) characterize the molecular signature of this lymphoma entity, (2) explore some signaling pathways implicated in its pathogenesis, and (3) search for novel markers potentially useful for diagnosis and/or targetable by therapeutic agents.

PATIENTS, MATERIALS AND METHODS

Patient characteristics and tumor samples

Nine newly diagnosed, previously untreated NKTCL patients with high quality RNA and/or DNA extracted from frozen tumor biopsies containing more than 60% tumor cells were selected for this study. Their main clinical, phenotypic and molecular characteristics are summarized in Table 1. Seven tumors originated in the nasopharyngeal area, one in the skin, and one in the hypophysis. All cases were reviewed by three hematopathologists (L.d.L, Y.H. and P.G.) and diagnosed according to the WHO criteria.[1] All cases had a CD3+ (cytoplasmic), CD2+, CD7+, CD5-, CD4-. CD8-, TiA1+, gzm B+ phenotype. Eight cases were positive for EBV by in situ hybridization with EBERs probes. A high load of EBV DNA was demonstrated by PCR in the remaining case. Specimens were also investigated for TCR γ chain gene rearrangement using a GC-clamp multiplex polymerase chain reaction (PCR)-γ-DGGE procedure. Seven cases without clonal T-cell population were regarded as of NK-cell origin, and a cytotoxic T-cell derivation was established in two cases with a clonal TCR gene

rearrangement. For immunohistochemical validation, formalin-fixed, paraffin-embedded tumor samples from 16 NKTCL, including seven of the cases described above, and 17 PTCL, NOS were selected.

The present study was approved by the institutional review board "Comité de Protection des Personnes Ile de France IX", Créteil, France.

Cell lines and normal NK cells

Two NKTCL cell lines, SNK6 and SNT8 (Table 1),[16] and two samples of normal CD56+ NK cells purified from peripheral blood including resting and IL-2-activated NK cells, were also subjected to gene expression profiling. The MEC04 NKTCL cell line was also used for *in vitro* proliferation assay and immunohistochemical validation.[17] U937, a myelomonocytic cell line (ATCC LGC Standards, Molsheim, France), was used as control for *in vitro* assay. Cell lines were cultured in RPMI 1640 supplemented with 2 mM L-glutamine and 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA) in the presence of 100 U/ml recombinant human IL-2, except for U937 cell line. Centrifugated pellets of SNK6 and MEC04 cells were fixed in ethanol to construct paraffin-embedded blocks.

RNA and DNA extraction

Total RNA was extracted with TRIZOL reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions, and DNA was extracted with phenol-chloroform using standard procedures. The integrity of the extracts was verified on an Agilent 2100 Bioanalyser (Agilent Technologies, Palo Alto, CA, USA).

Microarray procedures

Microarray analyses were performed using 3 μg total RNA as starting material and 10 μg cRNA per hybridization (GeneChip Fluidics Station 400; Affymetrix, Santa Clara, CA). The total RNAs were amplified and labeled following the one-cycle target labeling protocol (http://www.affymetrix.com). The labeled cRNAs were hybridized to HG-U133 plus 2.0 Affymetrix GeneChip arrays (Affymetrix, Santa Clara, CA). The chips were scanned with an Affymetrix GeneChip Scanner 3000 and subsequent images analyzed using GCOS 1.4 (Affymetrix).

Gene expression analyses

The gene expression analysis encompassed HG-U133 plus 2.0 Affymetrix array data from seven NKTCL biopsies, two NKTCL cell lines (Table 1), 16 PTCL, NOS recently reported by our group under accession number E-TABM-702,[18] six normal NK cells samples (including four previously published from GSE8059),[19] eighteen recently published normal B cell samples (eight from GSE15271 and 10 from GSE12195),[20,21] and 15 activated B-cell-like diffuse large B-cell lymphoma (ABC-DLBCL) samples from GSE12195.[21] Two NK cell samples without stimulation of IL-2 (one each from ours and from GSE8059) represented the "resting NK cell" group, while the two NK cell samples stimulated by IL-2 for 24 hours (one from our sample, the other from GSE8059) were regarded as the "activated NK cell" group. Affymetrix raw data of the samples were normalized in batch using Robust Multichip Average (RMA) method along with 58 other samples unrelated to this study. The clustering analysis of the 25 tumor samples was performed as already described.[22] To identify genes differentially expressed between two groups of samples we used Welch's T-tests. To control for multiple testing, we measured the local false discovery rate using kerfdr R package, an alternative approach to the more commonly used Bonferroni and Benjamini-Hochberg methods.[23] The KEGG / Biocarta pathways enrichment scores were calculated by combining

four methods including Significance Analysis of Microarray to Gene-Set Analysis (SAM-GS), Gene Set Analysis (GSA), and globaltest. The median score across the four methods was used to rank the pathways. Methodological details are given in the Supplementary Materials and Methods (Method S1). Raw gene expression data have been deposited to ArrayExpress under accession number E-TABM-702.

Array-based comparative genomic hybridization (aCGH)

Genome analyses of eight NKTCLs, SNK6 and SNT8 cell lines were performed using the human genome-wide CIT-CGH array (V6) containing 4434 sequence verified bacterial artificial chromosome (BAC) clones (with quadruplicate spots per clone) with a median gap between two successive clones of 600 kb. The characteristics of this array have been previously reported. aCGH raw data (deposited to ArrayExpress under accession number E-TABM-791) were normalized using the within print-tip group lowess method; smoothing, breakpoints detection and status (Gain/Loss) determination were calculated using the Bioconductor package GLAD. Methodological details are given in the Supplementary Materials and Methods (Method S1).

Immunohistochemistry

Immunohistochemistry was performed on deparaffinized tissue sections using a standard indirect avidin-biotin immunoperoxidase method. After appropriate antigen retrieval, sections were stained for EBI3 (2G4H6);[24] pSTAT3 at Tyr705 (D3A7), pAKT at Ser473 (736E11) (Cell Signaling Technology, Danvers, MA); VEGFA (C1), VCAM-1 (E10), cRel (B-6), PDGFRα and pPDGFRα (Santa Cruz Biotechnology, Santa Cruz, CA); E-cadherin (NCH-38, DakoCytomation, Glostrup, Denmark); CD163 (10D6, Novocastra-Leica, Wetzlar, Germany); clusterin (41D, Upstate-Millipore, Billerica, MA); CCND1 (SP4, Lab Vision-Thermo Fisher

Scientific, Fremont, CA); epidermal growth factor receptor (EGFR) (31G7, Zymed-Invitrogen, Carlsbad, CA); β-catenin (14, BD Biosciences, Erembodegem, Belgium); RelB (EP613Y, Epitomics, Burlingame, CA). For granzyme H (gzm H) (4G5)[25] and RelA (Santa Cruz Biotechnology), tyramide signal amplification system (CSAII kit, DakoCytomation) and peroxidase-labeled destran polymer (EnVision+, DakoCytomation) were applied, respectively. Adequate control tissues for specific antibodies were included.

Quantitative reverse-transcriptase PCR analysis of candidate genes

The expression of candidate genes (*HACE1*, *CCL2*, *TNFRSF21*, *CCND3*, *MET*) identified in altered chromosomal regions was determined by TaqMan® quantitative reverse-transcriptase PCR (qRT-PCR) (Applied Biosystems, Foster City, CA) in six primary tumors, three NKTCL cell lines and in resting normal NK cells as control. The expression of *HACE1* was compared to that of *PRDM1*, *ATG5* and *AIM1*, three candidate tumor suppressor genes in NKTCL also mapping at 6q21.[5] All primers and probes were purchased from Applied Biosystems and the gene expression was measured using Mastercycler® ep realplex^{2S} system (Eppendorf, Hamburg, Germany). Quantifications were done in duplicate and mean values and standard deviation were calculated for each transcript as previously described.[26]

PDGFRA gene analysis

PDGFRA gene analysis was performed in six primary tumors and in three cell lines. *PDGFRA* copy numbers in intron 1 (Syst: Hs05935655_cn,hg18.v7) and exons 7-8 (Syst: Hs04818823_cn,hg18.v7) were measured on a LightCycler 480 (Roche Diagnostics, Meylan, France), using TaqMan® copy number assays according to the manufacturer's instructions (Applied Biosystems). *ALB* gene was used as the reference gene.[27]

The mutations of *PDGFRA* exons 12, 14 and 18 were searched by Length Analysis of PCR Products (LAPP) and sequencing as described previously.[28]

To search for mutations and polymorphisms in the *PDGFRA* promoter region, genomic DNA was amplified by PCR using the primers 1651F and 727R as described by Toepoel et al.[29]. After purification (PCR purification kit, GE Healthcare, Freiburg, Germany), automated sequencing was performed on the ABI310 genetic analyzer with the Big Dye terminator sequencing kit (Applied Biosystems) using the primers 1562F, 1340R, 1110F, and 759R (see supplementary data). Sequences were compared to the reference sequence for *PDGFRA* promoter (GenBank accession number X80389).

^{3}H thymidine-labeled proliferation assay

Cells were washed extensively to remove IL-2 and plated at 20,000 cells per well, in the presence or absence of imatinib mesylate (Axon Medchem BV, Groningen, The Netherlands), at 3 different concentrations (1, 3, and 6 μM) and incubated for 72 hours. One μCi of tritium thymidine was added 6 hours before evaluating the proliferation using Packard TopCount Liquid Scintillation Counter (GMI, Ramsey, MN). Results were expressed as the percentage of proliferation measured in the absence of inhibitor. All experiments were performed in triplicate.

RESULTS

The molecular signature of NKTCL differs from that of normal NK cells

The Affymetrix expression profiles of seven NKTCL samples were compared with those of normal NK cells (n=6). A total of 2447 and 2339 probe sets corresponding to 1721 and 1436 overexpressed and underexpressed genes, respectively, significantly distinguished NKTCL tissues from normal NK cells ($P < 0.005$) (Tables 2 and S1). As expected, genes related to the

microenvironment, notably macrophages (*CLU, CD68, CD163*) were among the most differentially overexpressed in NKTCL tissues. As shown in Tables 2 and S1, the other genes overexpressed in NKTCL tissues included genes involved in the autophagy (*ATG3, ATG7*) and in the cell cycle control (*CCND1*), related to cell-to-cell interactions (*CDH1, ITGA7, ITGA9, ITGB4, VCAM1*), chemokines (*CX3CL1, CXCL9, CXCL10*), cytokines (*IL8, IL20, IL33*), extracellular matrix (ECM) interactions (*MMP11, MMP14, TIMP1, TIMP2, TIMP3*), innate immunity (*IL4I1, TLR4, TLR7, TLR8*), local invasion and metastasis (*COL1A1, COL1A2, FN1, LAMB1*), angiogenesis (*ANGPT2, VEGFA, VEGFB, VEGFC, KDR*), growth factors and their receptors (*PDGFRA, PDGFB, PDGFC, TGFB2, TGFB3*), oncogenes (*MAFB, MET, MYC*) and genes reported to be induced by EBV in vitro (*CDH1, EBI3, BASP1, DNASE1L3, HCK, HLA-DQA1, IFI30, IFI44L, IFITM3, LGALS9, THRA, TNFRSF10D*).[15,30] *PCDH15*, a gene previously shown to be specifically expressed in NKTCLs[31] was also overexpressed, albeit with less statistical significance. Some of these genes were also overexpressed in SNK6 and SNT8 cell lines compared to normal NK cells, for example, *VCAM1, CXCL10, EBI3, TIMP1, MYC, PDGFRA, KDR* and *VEGFA* (Tables 2 and S1). Interestingly, NKTCL appeared to be more closely related to activated than to resting NK cells, as the level of expression of about two-thirds of the NKTCL signature was closer to that of activated NK cells than that of resting NK cells.

Among these overexpressed genes tested by immunohistochemistry, E-cadherin, CD163, clusterin, EGFR, and cyclin D1 expression was restricted to non-neoplastic cells, i.e. epithelial cells (E-cadherin, clusterin, cyclin D1, EGFR), stromal cells (clusterin, cyclin D1) and numerous histiocytes (CD163) (Table 3). By contrast, VCAM1 and the EBV-induced gene 3 (EBI3), a subunit of IL-27, labeled the neoplastic cells of 4/13 (31%) and 9/12 (75%) evaluable NKTCLs, respectively (Figure 1B). SNK6 and MEC04 cells were also positive for EBI3.

NKTCL has a distinct molecular signature compared to PTCL, NOS

Unsupervised, consensus clustering applied to the molecular signatures of nine NKTCL samples (seven biopsies and two cell lines) and 16 PTCL, NOS samples, generated two main branches correlated to the pathological diagnosis. Indeed, all NKTCL samples clustered in one branch, and the other branch comprised PTCL, NOS cases only (Figure 2). Interestingly, one case of nodal γδ PTCL, NOS with an activated cytotoxic profile (gzm B+) but negative for EBV (sample C2) clustered with NKTCL cases.

By supervised analysis, a total of 372 and 691 probe sets corresponding to 278 and 484 overexpressed and underexpressed genes, respectively, significantly distinguished tissues involved by NKTCLs versus PTCLs, NOS ($P < 0.005$) (Tables 4 and S2). As expected, among the top overexpressed genes in NKTCLs were many genes encoding KIRs, and others encoding NK-cell associated molecules (*NCAM1, CD244*) or related to cytotoxic functions (*GZMB, GZMH, CTSW, PRF1…).* Significantly, NKTCLs also overexpressed genes encoding cell adhesion molecules (*ITGAM, ITGB6*), chemokines and their receptors (*CCL4*, *CCL5*, *CCR1*), apoptosis-related molecules (*FASLG, BCL2L2*), cytidine deaminase (*APOBEC3G*), and oncogenes (*MYC, RHOC*). In addition to NCAM1 (CD56) and cytotoxic proteins such as TiA1 and gzm B which were strongly expressed in all NKTCLs, we investigated the expression of another cytotoxic granule-associated protein, gzm H, of which transcript levels were 13.66 times higher than in PTCLs, NOS. As shown in figure 1, a strong granular cytoplasmic staining was found in virtually all neoplastic cells of all NKTCL tumors tested (n=16) as well as in the SNK6 and MEC04 cells whereas gzm H was restricted to small lymphocytes in most PTCLs, NOS. Among PTCL, NOS, the γδ T-cell lymphoma was gzm H positive and another case disclosed heterogeneous partial staining.

Patterns of copy number aberrations and identification of genes relevant to the pathobiology of NKTCL

The aCGH findings are summarized in Table 5 and in Figure 3. Extensive losses and gains of larger and smaller chromosomal regions were found in all NKTCL samples analyzed. Recurrent copy number aberrations (CNA) observed in ≥ 3/8 (37.5%) NKTCL biopsy samples, comprised 16 regions of chromosomal gains (on 10p15, 7q35-q36, 7p11.2-p12, 7q11.2-q34, 16p13.3, 17q12 and on 1q21-q44, 4p16, 6p11.1-p25, 6q11.1-q14, 6q27, 8p23.3, 9q34, 10p14-p15, 11p15, and 22q11.21) and four regions of losses (on 6q16-q25, 11q24-q25, 17p13.3, and 8p22-p23).

To identify candidate genes in the regions of CNA, we selected the genes with a Welch's T-test *P*-value less than 0.001 and a fold average expression difference > 2.0 (or < 0.5) in NKTCL tissues compared to normal NK cells. The resulting list (Table S3) included genes related to malignant transformation and invasion (*S100A16*, *LAMB1*, *LAMC1*, *COL1A2*, *CTSB*), cell cycle progression (*CCND3*), signal transduction (*FYN)*, tumor suppressor genes (*HACE1*, *CAV1*, *CAV2*, *DLC1*), as well as members of important pathways which, according to GEP analysis, appear deregulated in NKTCL (see below), such as angiogenesis (*MET*, *S100A13*), NF-κB (*PRKCQ*, *TNFRSF21*), WNT (*CUL1*, *FZD1*, *SGK1)*, and JAK-STAT (*AKT3*, *IL6R*, *CCL2*) signaling pathways.

We selected for qRT-PCR correlation several biologically relevant deregulated candidate genes mapping to altered chromosomal regions at 6q21 (*HACE1)*, 17q12 (*CCL2)*, 6p12.3 (*TNFRSF21)*, 6p21.1 (*CCND3)*, 7q31.2 (*MET*). We also evaluated *ATG5*, *AIM1*, *PRDM1* genes reported as candidate tumor suppressor genes at 6q21 in NKTCL cell lines, that were downexpressed in our microarray data albeit at less statistical significance. In agreement with the microarray results, we confirmed by qRT-PCR the overexpression of *MET*, *CCL2*, *TNFRSF21* in NKTCL samples compared to normal NK cells and the

underexpression of *CCND3* and *HACE1* (Figure 4). *ATG5* and *AIM1* transcripts were also markedly reduced in both cell lines and primary tumors. In contrast, quantification of *PRDM1* mRNA gave discrepant results in cell lines (underexpressed levels, in accordance with published data[5]) and primary tumors (increased transcript levels in four of six samples). These findings were independent of the presence or absence of 6q21 deletion.

Identification of distinct pathways in NKTCL

In order to identify potentially relevant biological pathways in NKTCL, four gene sets analysis methods were applied. The most discriminatory pathways when comparing NKTCL and normal NK cells or PTCL, NOS, comprised those related to important cell functions such as apoptosis, cell adhesion, cell communication, ECM-receptor interaction, cell cycle (p27 regulation), cytokine-cytokine receptor interaction, as well as TGFβ, MAPK, WNT and JAK-STAT signalling pathways (Table 6, Figure 5A and E). More specifically, angiogenesis-related (VEGF) and NF-κB were also discriminatory pathways between neoplastic samples and normal NK cells. FAS and AKT signaling genes pathways and especially genes related to NK-cell mediated cytotoxicity (Figure 5B, C, D, and F) were differentially expressed between NKTCL and PTCL, NOS.

For example, as illustrated in Figure 5A, several component genes of JAK-STAT pathway like *IL13RA1, IL6R, STAT1, STAT2, VEGFA, CCND1* and *MYC* were overexpressed in NKTCL compared to normal NK cells and *IL12RB1, IL20, IL2RB,* and *MYC* compared to PTCL, NOS. Altogether, the pathway analysis supports the involvement of several important pathways in NKTCL.

Activation of several oncogenic pathways (AKT, STAT3, NF-κB, VEGF) in NKTCL

In order to evaluate the possible activation of pathways identified using statistical methods, the expression of several representative genes was assessed at the protein level (Table 3 and Figure 1). Nuclear expression of phosphorylated AKT (Ser473) and STAT3 (Tyr705) was found in 9/9 and 13/14 NKTCL cases, respectively. Strong expression of VEGFA, one of the important STAT3-regulated gene products related to angiogenesis and immune evasion,[32] was demonstrated in 9/9 NKTCL and expression of its receptor VEGFR2 was evidenced in the neoplastic cells of 4/5 tumors. Positivity for pSTAT3 and VEGFA was confirmed in MEC04 and SNK6 cell lines, and pAKT was demonstrated in MEC04 cells.

Differential expression of WNT and NF-κB pathway genes were also identified in pathway analyses. Nuclear expression of β-catenin, a hallmark of activation of WNT pathway, was not observed in neoplastic cells (n=8), contrasting with cytoplasmic and/or membrane staining in surrounding epithelial and endothelial cells. On the other hand, RelA and cRel, two important molecules of the canonical NF-κB pathway, were expressed in the cytoplasm of most evaluable NKTCLs associated with nuclear localization of RelA (Figure 1). RelB, a molecule of the alternative NF-κB pathway, was negative in all investigated NKTCLs (n=6).

Activation of PDGFRA pathway in NKTCL

Since PDGFRα, a receptor tyrosine kinase mediating important cell functions such as migration, proliferation and cell survival and known to interact with PI3K/AKT and STAT proteins,[33] was highly expressed at the mRNA level (Table 2 and Figure 5B), this factor was evaluated by immunohistochemistry. Eleven of 13 (85%) NKTCL cases showed cytoplasmic expression of PDGFRα. In addition, its phosphorylated form (pPDGFRα) was found in all 13 NKTCLs (Table 3 and Figure 1). In agreement with a recent report,[34] expression of both PDGFRα and pPDGFRα was also demonstrated in most PTCLs, NOS (Table 3). In view of

these findings, we investigated the potential implication of the PDGF signaling pathway in the proliferation of NKTCL cells. Imatinib mesylate induced dramatic concentration-dependent growth inhibition of PDGFRα-positive MEC04 cells whereas the effect on SNK6 cells was minimal. As shown in Figure 6, a significant 50% growth inhibition was already observed when MECO4 cells were exposed to imatinib mesylate at 1µM that increased to 90% at 6 µM. U937 used as a control cell line known to be resistant to imatinib mesylate maintained 63 % proliferation with 6 µM imatinib.

We further searched for potential genetic alterations underlying *PDGFRA* deregulation. The absence of copy number gain in *PDGFRA* locus validated by real-time PCR in eight interpretable samples was concordant with the absence of genomic imbalance in 4q11-q13 observed in our BAC array CGH data. In addition, we did not evidence mutations in exons 12, 14 and 18 of *PDGFRA*, in agreement with previous findings.[35]

Finally, we performed mutations and polymorphisms analysis within the *PDGFRA* promoter region and in particular investigated the distribution of haplotypes which have been reported in association with different transcriptional activities.[36] Sequence analysis did not show any mutation in the nine samples tested (compared to the reference sequence), and the distribution in the haplotypes - four samples had a H1/H2α genotype, three H2α/H2α, one H2α/H2β and one H2α/H2γ – corresponded to that reported in the Western European population (see supplementary data).

DISCUSSION

In the present study, we characterized the molecular signature of NKTCL in comparison to normal NK cells and to PTCL, NOS. This led to the identification of deregulated genes and signaling pathways which might be relevant to the pathophysiology and clinicopathologic features of the disease and bring rationale for the development of new therapies.

Unsupervised clustering remarkably segregated NKTCL and PTCL, NOS samples. Notably, the NKTCL case with a T-cell cytotoxic phenotype (sample T5) clustered with those of NK cell origin, providing another molecular argument for grouping nasal "true NK-" and cytotoxic T-cell lymphomas as a single entity as proposed in the current WHO classification.[1] Interestingly, the NKTCL case of T-cell derivation also showed the 6q16-q25 deletion. The only mismatch was represented by one PTCL, NOS in the NKTCL cluster. That particular case had a γδ activated cytotoxic phenotype suggesting that derivation from the innate immune system might imprint a peculiar signature.[37]

By comparison to PTCL, NOS, the molecular signature of NKTCL was significantly contributed by an overexpression of genes associated with cytotoxic functions and NK-cell-associated molecules. Interestingly, the highest fold change of expression was observed for gzm H transcripts. Granzyme H, a gzm family member sharing a 90% amino acid sequence identity with gzm B, is constitutively expressed in NK cells irrespective of their activation status[25] but acts differently from gzm B by inducing a caspase-independent cell death program.[38] We confirmed a strong gzm H protein expression in all NKTCLs contrasting with its negativity in most PTCLs, NOS, with the notable exception of the γδ T-cell lymphoma. Therefore, gzm H appears to be a novel sensitive marker for NKTCL, although its specificity needs to be delineated with respect to its possible expression in other lymphomas derived from the innate immune system.

Other aspects of the NKTCL signature could be related to some peculiar clinicopathologic features of the disease. Angioinvasion and angiocentricity typical of NKTCL, might be accounted by the high expression of genes such as *VCAM1*, *CXCL9,* and *CXCL10,* encoding proteins involved in the interaction with endothelium or in the pathogenesis of tissue necrosis and vascular damage associated with EBV-positive lymphoproliferations.[39] NKTCL, which in most instances arises in the nasal area, is also

characterized by a strong tendency to disseminate to other extranodal distant sites. In view of the known roles of CCR7 and SELL/CD62L in peripheral lymph node homing and that of CCL27, CXCL12, in homing to the skin, intestine and bone marrow, it is likely that the lower levels of CCR7 and SELL/CD62L and the overexpression of CXCL12 might explain the pattern of distribution of the disease.

We confirmed here recurrent genomic gains and losses previously reported in NKTCL. Deletion of chromosome 6q reported as the most characteristic but not specific genetic alteration in NKTCL,[3-5,40-42] was present in 40% of our cases, including SNK6 cell line. In line with the recent report by Iqbal et al., we also found recurrent gain of 1q21-q44 and loss of 17p13.3 in primary tumor samples.[5] These authors found *PRDM1*, *ATG5*, and *AIM1* as target genes in the region of del6q21 and reported both mutation and methylation in *PRDM1*. Here, we further extended these previous findings by showing low levels of *ATG5* and *AIM1* transcripts in primary tumors. Conversely, *PRDM1* showed a wide range of expression from case to case. In addition, we also found marked reduction in transcripts of *HACE1*, a gene encoding a novel E3 ubiquitin ligase, which is the target of epigenetic inactivation in Wilms' tumor and has been proposed as a tumor suppressor gene in multiple human cancers. *Hace1*$^{-/-}$ mice are spontaneously prone to developing multiple malignant tumors in various organs.[43] Taken together, it is therefore tempting to speculate that *HACE1* might be also involved in the pathogenesis of NKTCL.

The proto-oncogene *MET* mapping in 7q31, a region of recurrent gain in our series, was overexpressed in our samples. This receptor with tyrosine-kinase activity is a receptor to HGF, also overexpressed at the mRNA level in our NKTCL series. Interestingly, this pair of ligand-receptor appears to be linked to angiogenesis, tumor formation, invasion, and metastasis.[44] These findings, together with the expression of VEGFA and its receptor

VEGFR2 might reflect the implication of angiogenesis and/or VEGF signaling pathway in the pathophysiology of NKTCL.

The molecular pathways involved in the pathogenesis of NKTCL are largely unknown. Our study identified deregulated pathways in NKTCL, in comparison to normal NK cells and PTCL, NOS. Among growth factor receptors, the receptor tyrosine kinase PDGFRα was expressed at a higher level than in normal NK cells, both at the mRNA and protein levels, in its activated phosphorylated form, a feature which appears to be shared with PTCL, NOS.[34] PDGF signaling pathway is known to be associated with both JAK-STAT and AKT pathways. The AKT protein kinases play a critical role in cell proliferation, survival and programmed cell death, transcription, and cell migration via phosphorylation of a multitude of substrates. Signal transducers and activators of transcription (STATs) are transcription factors activated in response to cytokines and growth factors. STAT3 in particular plays a crucial role in regulating cell growth and apoptosis.[32] Several solid or hematological malignancies including ALK-positive anaplastic large cell lymphomas show constitutive STAT3 activation. Using statistical methods, we showed here that AKT and JAK-STAT pathways were differentially expressed in comparison to normal NK cells and/or PTCL, NOS. In addition, we evidenced the nuclear expression of the phosphorylated forms of STAT3 and AKT in most NKTCLs implying constitutive activation of these pathways in this disease. Our results expand recent findings that AKT was phosphorylated in NK-92 cell line and in a few NKTCL primary tumors, probably through involvement of IL-2 or IL-15.[45] Many genes related to proliferation and survival, angiogenesis, and immunosuppression are known to be regulated by STAT3.[32] The high transcript levels of several genes regulated by STAT3 in NKTCL compared to normal NK cells such as *MYC, VEGFA, BCL2L1,* - and also less significantly *BIRC5, HGF, IL6, MMP2, MMP9, IL10,* and *CDK5* - suggests its implication in the pathogenesis of NKTCL. Among these, MMP2, MMP9 and IL-10 proteins have already been

evidenced in NKTCL tumor cells and we show here immunohistochemical expression of VEGFA.[46,47] Recently, we demonstrated that the inhibition of STAT3 activation leads to underexpression of two STAT3 target gene products, BCL-X_L and MYC in MEC04 cells.[17] Altogether, these data provide strong arguments supporting the involvement of JAK-STAT and AKT pathways in NKTCL (Figure 7).

EBV, constantly present in NKTCL, is suspected to play an important role in oncogenesis. Here we showed that, in comparison to EBV-negative normal NK cells, NKTCL overexpressed several EBV-induced genes.[15,30] In agreement with a previous report,[24] one of these genes, *EBI3,* was validated at the protein level. Among the various mechanisms involved in NF-κB pathway, EBV is known to activate NF-κB pathway through LMP-1 and/or TRAF,[48] especially in Hodgkin lymphoma and in EBV-positive B-cell lymphoproliferative disorders. Here, we showed differential expression of this pathway in NKTCL and further demonstrate expression of RelA, supporting activation of NF-κB in this entity. Interestingly, *TNFAIP3* gene, encoding an inhibitor of the NF-κB pathway, maps to the region of recurrent loss in 6q16-q25 and was underexpressed in our study. Deletions and/or somatic mutations of this gene have been recently reported in classical Hodgkin lymphoma and primary mediastinal B-cell lymphoma as well as in MALT lymphoma[49,50] supporting the role of this key regulator of NF-κB activity as a novel tumor suppressor gene in these lymphomas. Altogether, these findings suggest the involvement of NF-κB pathway in the pathogenesis of NKTCL. The respective role of EBV and/or *TNFAIP3* inactivation in NF-κB activation in NKTCL needs further investigation.

The demonstration of pPDGFRα by immunohistochemistry prompted us to test the sensitivity of NKTCL cell lines *in vitro* to imatinib mesylate. The effect of the drug already significant at a 1µM concentration with a 50% growth inhibition on MEC04 cells, was most prominent (90% inhibition of growth) at 6µM. Conversely, there was no substantial cytotoxic

effect on the SNK6 cell line, which might be related to a lower expression of *PDGFRA*, as suggested at the RNA level (Figure 3). Although our results do not preclude the precise mechanism of action of imatinib and suggest heterogeneity in the sensitivity to the drug, the dramatic effect on MEC04 cells shed light on the possible use of tyrosine kinase inhibitors as a novel therapeutic option in some patients with NKTCL refractory to conventional therapies.

The cause of *PDGFRA* deregulation in NKTCL remains to be determined. We did not evidence either genomic imbalances or gene mutations. Furtheremore, mutations in the promoter region were absent in NKTCL primary tumors and cell lines, and we did not find overrepresentation of H2α haplotype, known to result in upregulation of *PDGFRA* in glioblastoma.[29]

In conclusion, this integrative genomic and transcriptomic study characterizes the molecular signature of NKTCL, highlights emerging oncogenic pathways in this disease entity and offers rationale for exploring new therapeutic options such as tyrosine kinase inhibitors in patients with this aggressive malignancy.

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Table 1. Summary of immunohistochemical, and genotypic features of the NKTCL samples analysed in the study.

Samples	Age	Sex	Biopsy site	CD3ε	CD2	CD5	CD7	CD4	CD8	CD56	TiA1	Gzm B	EBV	T clonality
T1	58	M	Nasopharyngeal region	+	+	-	+	-	-	+	+	+	+	-
T2	25	M	Nasopharyngeal region	+	+	-	-	-	-	+	+	+	+	-
T3	64	M	Skin	+	-	-	ND	-	-	-	+	+	+	-
T4	62	M	Nasopharyngeal region	-	+	-	-	-	-	+	+	+	+	-
T5	72	M	Nasopharyngeal region	+	+	-	-	-	-	+	+	+	+	+
T6	52	F	Nasopharyngeal region	+	+	-	-	ND	-	+	+	+	+	-
T7*	42	M	Hypophysis	+	+	-	-	+	-	+	+	+	+	-
T8†	31	F	Nasopharyngeal region	+	+	-	+	+	ND	+	+	+	+	-
T9†	67	M	Nasopharyngeal region	+	+	-	+	-	+	+	+	+	+	+‡
SNK6	NA	NA	NA	+	+	ND	ND	-	-	+	ND	+	+	-
SNT8	NA	NA	NA	+	+	ND	ND	-	-	+	ND	ND	+	+

*: sample used only for transcriptomic analysis.
†: samples used only for genomic analysis.
‡: this case showed βF1 immunoreactivity on formalin-fixed paraffin-embedded tissues sections, and was negative for δTCR1 on frozen sections.
NA: not applicable.
ND: not done.

Table 2. Selection of genes overexpressed in NKTCL tissues compared to normal NK cells with *P*-value less than 0.005. A complete list of genes is given in the supplementary table S1.

Gene class and specific genes	Fold change
Cell-to-cell interactions	
CDH1 (E-cadherin)*	1.54
ITGA9 (Integrin alpha 9)	2.37
ITGB4 (Integrin beta 4)*	2.15
VCAM1 (Vascular cell adhesion molecule 1)*	26.18
Cell cycle control	
CCND1 (Cyclin D1)*	6.67
Chemokines	
CCL18	10.50
CCL19	80.67
CCL2*	88.11
CCL8*	51.61
CXCL10 (IP10)*	48.07
CXCL12 (SDF-1)	25.75
CXCL9 (Mig)	254.14
Cytokines	
IL13RA1*	9.19
IL6R	2.25
IL33*	2.75
IL8*	5.07
Genes overexpressed in EBV-infected lymphoma cell lines	
EBI3 (EBV induced gene 3)*	2.76
BASP1 (Brain abundant, membrane attached signal protein 1)*	13.21
HCK (Hemopoietic cell kinase)	16.01
TNFRSF10D (Tumor necrosis factor receptor superfamily, member 10d)*	1.53
Extracellular matrix interactions	
COL1A1 (Collagen, type I alpha 1)*	45.91
COL1A2 (Collagen, type I alpha 2)*	33.35
COL3A1 (Collagen, type III alpha 1)*	59.68
FN1 (Fibronectin 1)*	91.79
TIMP1 (TIMP metallopeptidase inhibitor 1)*	6.28
TIMP2 (TIMP metallopeptidase inhibitor 2)	10.79
TIMP3 (TIMP metallopeptidase inhibitor 3)	9.57
Growth factors & receptors	
GHR (Growth hormone receptor)*	1.35
PDGFB (Platelet-derived growth factor beta)*	2.16
PDGFC (Platelet derived growth factor C)*	7.92
PDGFRA (Platelet-derived growth factor receptor, alpha polypeptide)*	7.47
Immunity & lymphocyte development	
CD86*	6.04
HLA-DOA*	12.12
HLA-DRA*	12.18
IL4I1 (Interleukin 4 induced 1)*	6.40
SLAMF8 (SLAM family member 8)*	45.26
TLR2	8.76
TLR7*	5.26
TLR8	13.49
Microenvironment	
CD163	134.47
Oncogenes	
MAFB (V-maf musculoaponeurotic fibrosarcoma oncogene homolog)	37.94
MET (Hepatocyte growth factor receptor))*	2.00
MYC*	1.23
Transcription factors	
STAT1*	5.25

STAT2*	1.86
Vascular biology	
KDR (VEGFR2)*	1.47
THBD (Thrombomodulin)	4.33
VEGFA*	1.54
VEGFC*	1.68
VWF*	12.97
Miscellaneous	
AIM2 (Absent in melanoma 2)*	14.76
CD40 (TNF receptor superfamily member 5)*	3.95
FZD1 (Frizzled homolog 1)*	2.55

*: genes are also overexpressed in NKTCL cell lines compared to normal NK cells.

Table 3. Summary of immunohistochemical results*.

	NKTCL (n=16)	NKTCL cell lines (n=2)	PTCL, NOS (n=17)
Clusterin	0/4 (0%)	0/1	ND
E-cadherin	0/4 (0%)	ND	ND
Cyclin D1	0/4 (0%)	ND	ND
EGFR	0/4 (0%)	ND	ND
CD163	0/4 (0%)	0/2	ND
EBI3	9/12 (75%)	2/2	1/13 (8%)
VCAM1	4/13 (31%)	0/2	0/7 (0%)
Granzyme H	16/16 (100%)	2/2	2/16 (13%)
pSTAT3	13/14 (93%)	2/2†	2/17 (12%)
pAKT	9/9 (100%)	NE	0/16 (0%)
VEGFA	9/9 (100%)	2/2	5/13 (38%)
VEGFR2	4/5 (80%)	2/2‡	ND
PDGFRα	11/13 (85%)	1/1	10/10 (100%)
pPDGFRα	13/13 (100%)	ND	7/8 (88%)
RelA	5/6 (83%)§	2/2§	ND
RelB	0/6 (0%)	ND	ND
cRel	4/5 (80%)‖	0/2	ND
Beta-catenin	0/8 (0%)	ND	ND

*: Results refer to expression in neoplastic cells.
†: phosphorylated STAT3 stained most MEC04 cells, but only a minority of SNK6 cells.
‡: performed by flow cytometric immunophenotyping.
§: cytoplasmic and nuclear staining of neoplastic cells.
‖: cytoplasmic staining of most neoplastic cells.
ND: not done.
NE: non evaluable.

Table 4. Selection of genes overexpressed in NKTCL tissues compared to PTCL, NOS cells with *P*-value less than 0.005. A complete list of genes is given in the supplementary table S2.

Gene class and specific genes	Fold change
Apoptosis	
BCL2L2 (BCL-W)	1.68
FASLG (Fas ligand)	5.99
Cell-to-cell interactions	
ITGAM (Integrin, alpha M)	2.30
Chemokines	
CCL4 (MIP-1-beta)	4.30
CCL5 (RANTES)	8.66
Cytokines & receptors	
IL20	1.15
IL2RB	2.32
Growth factors	
TGFB2	1.14
Cytotoxic molecules	
CTSW (Cathepsin W)	11.31
GZMA (Granzyme A)	8.03
GZMB (Granzyme B)	5.39
GZMH (Granzyme H)	13.66
PRF1 (Perforin 1)	3.34
NK cell-associated molecules	
APOBEC3G (Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G)	2.40
CD244 (NK cell receptor 2B4)	6.75
KIR2DL3 /// KIR2DS5	10.30
KIR2DL4	11.77
KIR2DL5A	7.47
KIR2DS4	4.94
KIR3DL1	9.96
KIR3DL2	14.02
KIR3DL3	12.51
KLRC1 /// KLRC2	25.75
KLRC3	32.96
KLRC4 /// KLRK1	10.33
KLRD1	32.45
NCAM1 (CD56)	10.54
Immune responses	
IRF6 (Interferon regulatory factor 6)	1.54
Oncogenes	
MYC	1.18
RHOC (Ras homolog gene family, member C)	3.22
Transcription factors	
ATF6 (Activating transcription factor 6)	1.39
Miscellaneous	
MAPK1	1.46
PTPN22 (Protein tyrosine phosphatase, non-receptor type 22)	2.47
YWHAH (Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, eta polypeptide)	1.62

Table 5. Copy number alterations identified by aCGH in the NKTCL samples analysed in this study.

	Chromosomes	Start position (Mb)	End position (Mb)	No of clones	NKTCL tissues and cell lines (n=10)	NKTCL tissues (n=8)	SNK6	SNT8
GAIN	**1q21-q44**	143	245	170	5	3	1	1
	4p16	2	6	5	3	3	0	0
	6p25-p11.1	0	58	93	4	3	1	0
	6q11.1-q14	58	87	26	4	3	1	0
	6q27	167	171	5	3	3	0	0
	7p12-p11.2	53	55	4	5	4	0	1
	7q11.2-q34	62	142	99	5	4	1	0
	7q35-q36	143	156	11	6	5	1	0
	8p23.3	0,13	1,96	5	4	3	1	0
	9q34	134	138	7	4	3	1	0
	10p15	0	4	5	6	6	0	0
	10p15-p14	6	8	6	3	3	0	0
	11p15	0	3	10	4	3	1	0
	16p13.3	1	2	5	4	4	0	0
	17q12	28,81	30,78	12	5	3	1	1
	17q12	29,84	30,30	7	6	4	1	1
	22q11.21	16,15	21,17	6	4	3	0	1
LOSS	**6q16-q25**	100	154	75	4	3	1	0
	8p23-p22	4	15	14	3	3	0	0
	11q24-q25	128	133	8	4	3	0	1
	17p13.3	0,34	0,96	6	4	3	1	0

Table 6. Selection of pathways differentially expressed in NKTCL tissues compared to normal NK cells or PTCL, NOS*.

Pathway name	Median *P*-value (compared to normal NK cells)	Median *P*-value (compared to PTCL, NOS)
Apoptosis		
Apoptosis	0.002	< 0.002
Regulation of BAD phosphorylation	0.109	< 0.018
PTEN dependent cell cycle arrest and apoptosis	0.246	< 0.015
FAS signalling pathway	0.257	< 0.006
Cytotoxicity		
Natural killer cell mediated cytotoxicity	0.002	< 0.0001
Ras-Independent pathway in NK cell-mediated cytotoxicity	0.325	< 0.0001
Cell adhesion molecules / extracellular matrix receptor interaction		
Cell communication	0.001	< 0.016
ECM receptor interaction	0.001	< 0.016
Focal adhesion	0.001	< 0.023
Cell adhesion molecules	0.002	< 0.012
Leukocyte transendothelial migration	0.002	0.371
Inhibition of matrix metalloproteinases	0.003	0.018
Integrin signalling pathway	0.003	0.484
Cell cycle		
Regulation of p27 during phosphorylation	< 0.006	0.008
Cyclin E destruction pathway	< 0.041	0.003
Chemokines / cytokines		
Cytokine cytokine receptor interaction	0.002	< 0.001
Signal transduction		
JAK-STAT signaling pathway	0.002	0.01
MAPK signaling pathway	0.002	0.015
mTOR signaling pathway	0.002	0.06
NF-κB signaling pathway	0.002	0.06
TGFβ signaling pathway	0.002	0.019
VEGF signaling pathway	0.002	0.418
Notch signaling pathway	< 0.005	0.025
Wnt signaling pathway	< 0.005	< 0.013
AKT signalling pathway	0.14	< 0.001

***:** with a median *P*-value < 0.02 compared to either normal NK cells or PTCL, NOS.

FIGURE LEGENDS.

Figure 1. Validation of gene expression profiling by immunohistochemistry. Representative NKTCLs disclosed cytoplasmic staining of neoplastic cells for (A) gzm H, (B) EBI3, (C) VEGFA, nuclear localization of (D) pSTAT3, (E) pAKT, cytoplasmic and nuclear staining of (F) RelA, and positivity for (G) PDGFRα and (H) pPDGFRα. Images were captured with a Zeiss Axioskop2 microscope (Zeiss, Oberkochen, Germany). Photographs were taken with a DP70 Olympus camera (Olympus, Tokyo, Japan). Image acquisition was performed with Olympus DP Controller 2002, and images were processed with Adobe Photoshop v7.0 (Adobe Systems, San Jose, CA). Original magnifications: x400 (A-H).

Figure 2. Unsupervised clustering of seven NKTCLs, two NKTCL-derived cell lines and 16 PTCLs, NOS. Dendrogram of 23 lymphoma tissues and two cell lines based on principal component analysis demonstrated two major clusters of NKTCL including tumor-derived cell lines and PTCL, NOS, respectively. T1-7: seven NKTCL samples, SNK6, SNT8, C1-16: 16 PTCL, NOS samples.

Figure 3. Genomic profiles of NKTCL. Pangenomic view of eight NKTCL tissue samples. The horizontal axis represents the genomic order, and the vertical axis represents the number of samples with gains or losses. Gains are represented as red bars and losses are represented as green bars.

Figure 4. Quantification of selected genes by qRT-PCR analysis. qRT-PCR analysis for (A) *MET*, (B) *TNFRSF21*, (C) *CCND3*, (D) *CCL2*, and (E) *PRDM1*, *ATG5*, *AIM1*, and *HACE1* in NKTCL primary tumors and cell lines. The results are expressed as relative fold change compared to resting CD3-/CD56+ NK cells sorted from peripheral blood. Quantifications were done in duplicate and mean values and standard deviation were calculated for each transcript. (A-D) In agreement with the microarray results, mRNA levels of *MET*, *CCL2*, *TNFRSF21* are increased in NKTCL primary tumors compared to normal NK

cells whereas *CCND3* mRNA is reduced (E) The analysis of four putative tumor suppressors in 6q21 region show that the transcripts levels of *HACE1, AIM1* and *ATG5* are reduced in primary tumors and cell lines whereas the mRNA level of *PRDM1* is increased in primary tumors, due to a wide variation from case to case (not shown).

Figure 5. Cellular programs deregulated in NKTCL. Representative molecular pathways differentially expressed in NKTCLs by comparison to either normal NK cells (A. JAK-STAT, B. angiogenesis, E. apoptosis), normal B cells and ABC-DLBCL (C. NF-κB), normal NK cells and normal B cells (D. AKT), or PTCL, NOS (F. cytotoxicity) were illustrated. For each line, green corresponds to the minimal intensity value (min), red corresponds to the maximal intensity value (max), and black corresponds to (min + max)/2. Several genes related to JAK-STAT, angiogenesis, and apoptosis-related pathways are overexpressed in NKTCLs compared to normal NK cells, and genes related to the cytotoxicity-related pathway are also overexpressed in NKTCLs compared to PTCL, NOS. Many genes in the NF-κB and AKT signaling pathways are overexpressed in NKTCLs compared to normal B cells, with a molecular signature similar to that of ABC-DLBCL in NK-κB pathway. The expression data of *PDGFRA* in NKTCLs, 2 NKTCL-derived cell lines, and normal NK cells is highlighted. T1-7: seven NKTCL samples, C1-16: 16 PTCL, NOS samples, AcNK, AcNK02, AcNK08, AcNK24: activated NK cells, ReNK, ReNK.: resting NK cells, CB.1-8 and CB.11: nine centroblasts samples, CC.1-8 and CC.11: nine centrocytes samples, ABC.2024-2195: 15 ABC-DLBCL samples.

Figure 6. Proliferation of NKTCL-derived cell lines in the presence of imatinib mesylate. SNK6, MEC04, and U937 cells were incubated for 72 hours in media with or without imatinib mesylate at concentrations of 1, 3, and 6 µM. Imatinib induced concentration-dependent growth inhibition of MEC04 cells. The effect on SNK6 was not significantly different from that observed on the myelomonocytic U937 cell line. The horizontal axis is the

concentration of imatinib mesylate. The vertical axis is the percentage of control proliferation. Bars indicate the SEM of triplicate experiments.

Figure 7. Hypothetical representation of signaling pathways involved in NKTCL. The interactions of PDGF, AKT, and JAK-STAT signaling pathways may contribute to the angiogenesis, immunosuppression, proliferation and survival of NKTCL.

Figure 1.

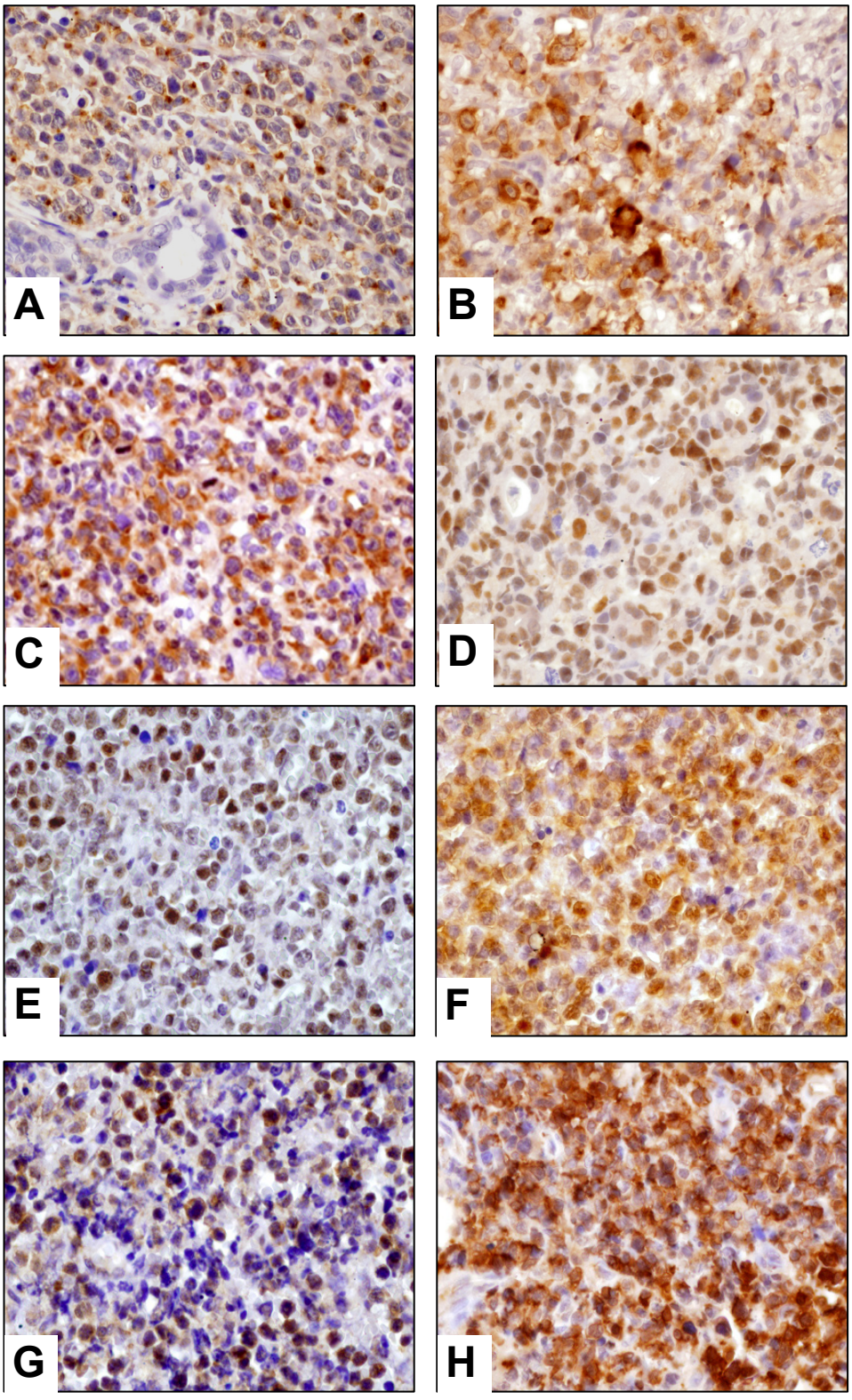


Figure 2.

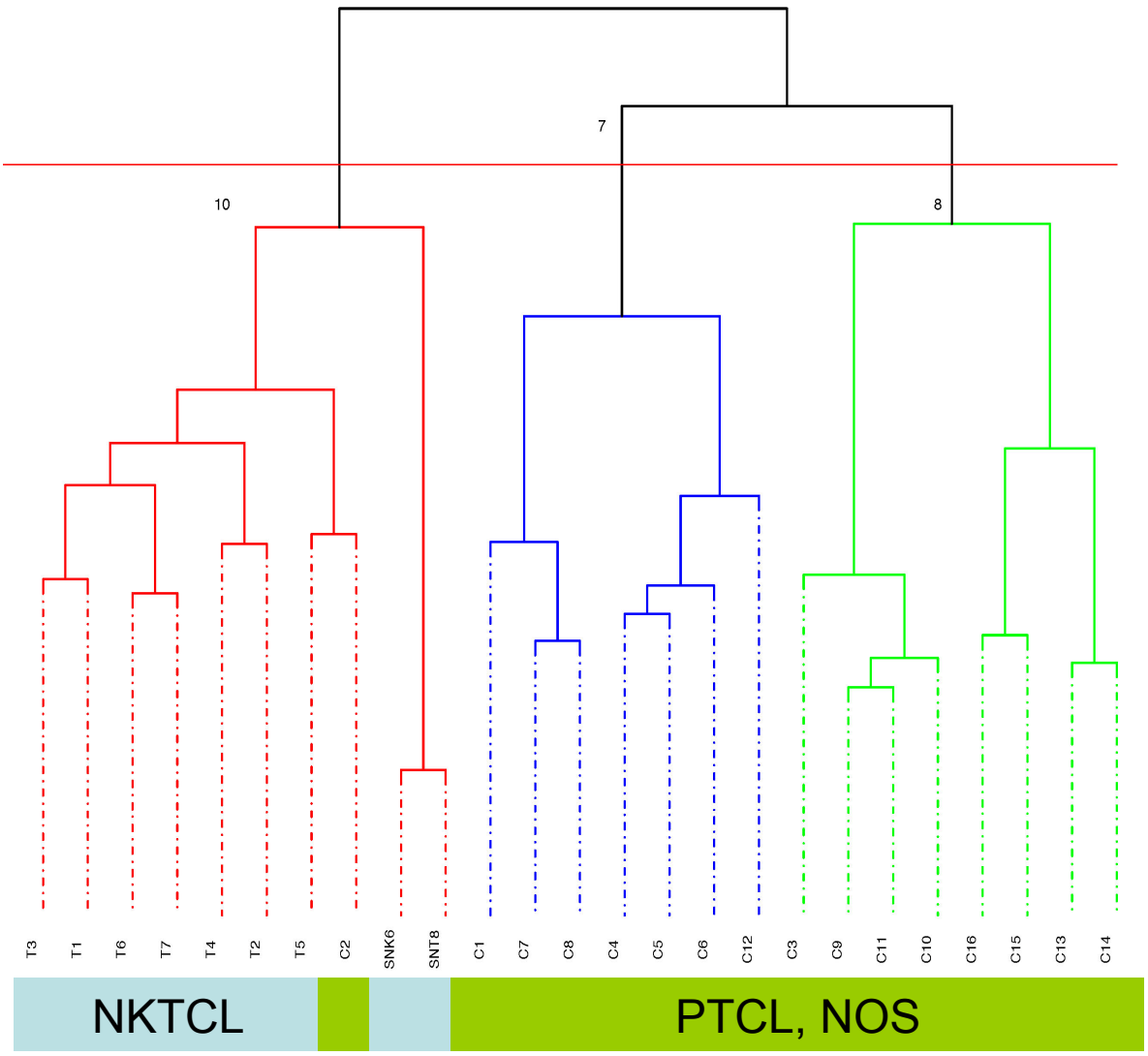

7
10
8
T3
T1
T6
T7
T4
T2
T5
C2
SNK6
SNT8
C1
C7
C8
C4
C5
C6
C12
C3
C9
C11
C10
C16
C15
C13
C14
NKTCL
PTCL, NOS

Figure 3.

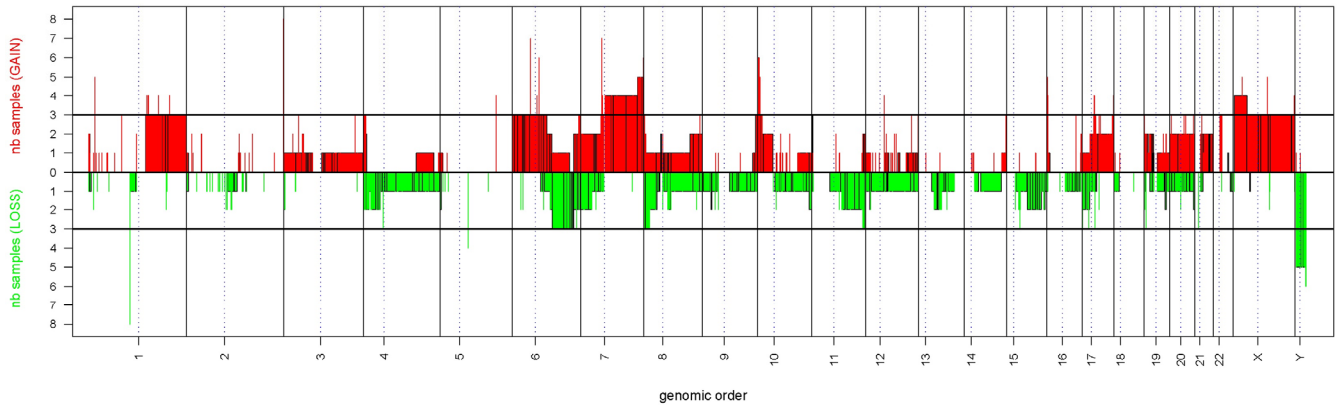

nb samples (GAIN)
nb samples (LOSS)
genomic order
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
X
Y

Figure 4.

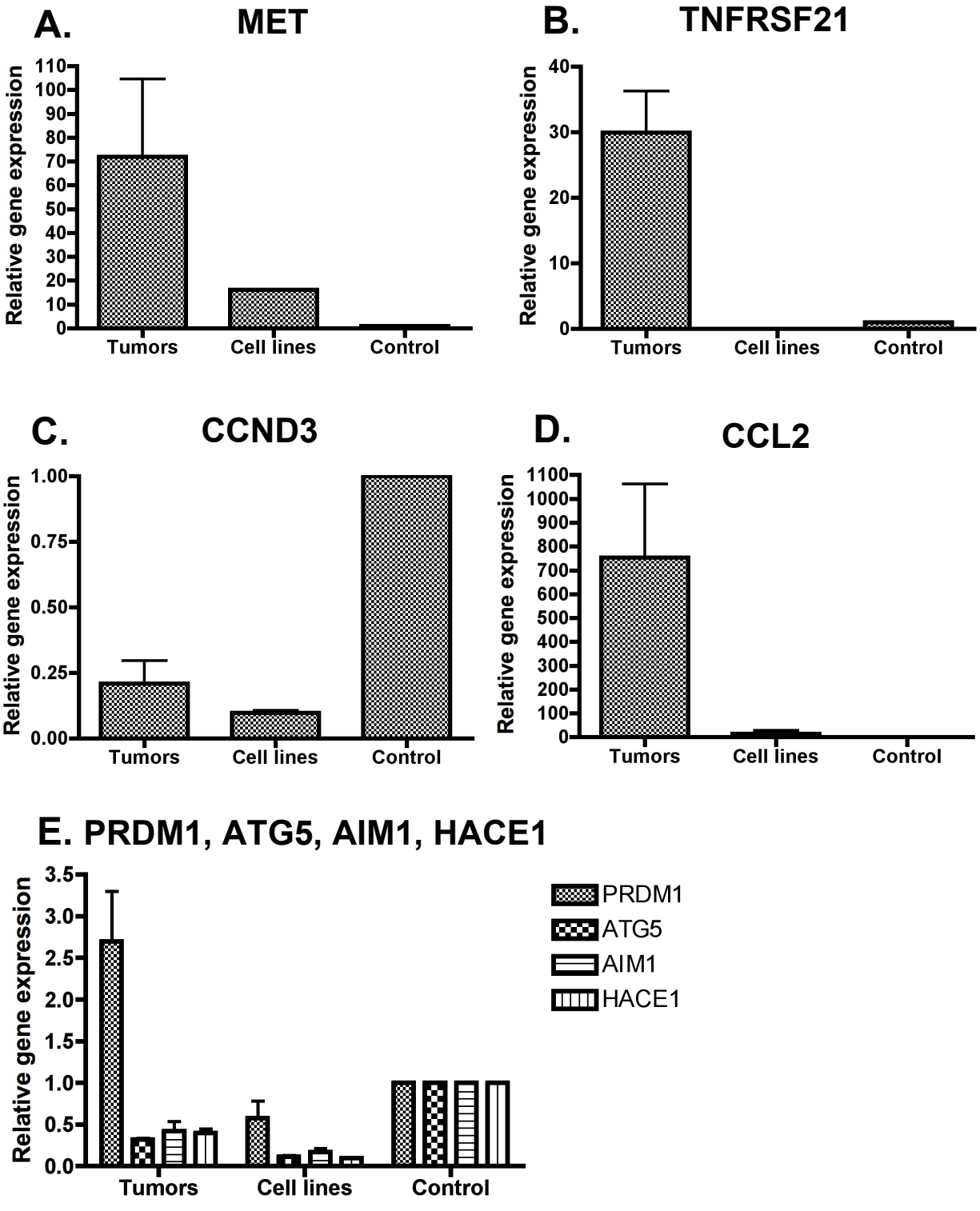

A.
MET
Relative gene expression
110
100
90
80
70
60
50
40
30
20
10
0
Tumors
Cell lines
Control
B.
TNFRSF21
Relative gene expression
40
30
20
10
0
Tumors
Cell lines
Control
C.
CCND3
Relative gene expression
1.00
0.75
0.50
0.25
0.00
Tumors
Cell lines
Control
D.
CCL2
Relative gene expression
1100
1000
900
800
700
600
500
400
300
200
100
0
Tumors
Cell lines
Control
E. PRDM1, ATG5, AIM1, HACE1
Relative gene expression
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Tumors
Cell lines
Control
PRDM1
ATG5
AIM1
HACE1

Figure 5.

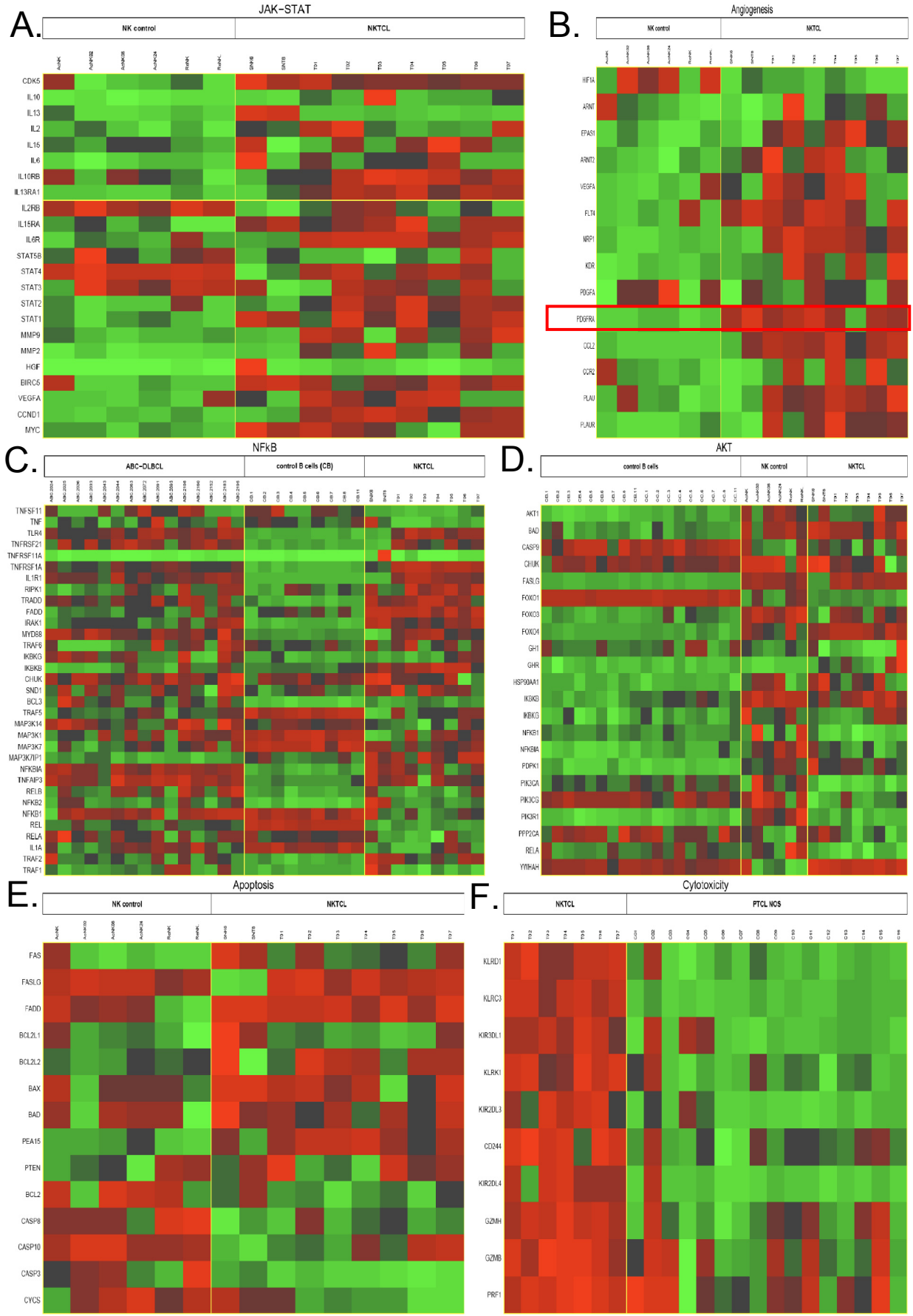

A.
JAK-STAT
NK control
NKTCL
CDK5
IL10
IL13
IL2
IL15
IL6
IL10RB
IL13RA1
IL2RB
IL15RA
IL6R
STAT5B
STAT4
STAT3
STAT2
STAT1
MMP9
MMP2
HGF
BIRC5
VEGFA
CCND1
MYC
B.
Angiogenesis
NK control
NKTCL
HIF1A
ARNT
EPAS1
ARNT2
VEGFA
FLT4
NRP1
KDR
PDGFA
PDGFRA
CCL2
CCR2
PLAU
PLAUR
C.
NFkB
ABC-DLBCL
control B cells (CB)
NKTCL
TNFSF11
TNF
TLR4
TNFRSF21
TNFRSF11A
TNFRSF1A
IL1R1
RIPK1
TRADD
FADD
IRAK1
MYD88
TRAF6
IKBKG
IKBKB
CHUK
SND1
BCL3
TRAF5
MAP3K14
MAP3K1
MAP3K7
MAP2K7IP1
NFKBIA
TNFAIP3
RELB
NFKB2
NFKB1
REL
RELA
IL1A
TRAF2
TRAF1
D.
AKT
control B cells
NK control
NKTCL
AKT1
BAD
CASP9
CHUK
FASLG
FOXO1
FOXO3
FOXO4
GH1
GHR
HSP90AA1
IKBKB
IKBKG
NFKB1
NFKBIA
PDPK1
PIK3CA
PIK3CG
PIK3R1
PPP2CA
RELA
YWHAH
E.
Apoptosis
NK control
NKTCL
FAS
FASLG
FADD
BCL2L1
BCL2L2
BAX
BAD
PEA15
PTEN
BCL2
CASP8
CASP10
CASP3
CYCS
F.
Cytotoxicity
NKTCL
PTCL NOS
KLRD1
KLRC3
KIR3DL1
KLRK1
KIR2DL3
CD244
KIR2DL4
GZMH
GZMB
PRF1

Figure 6.

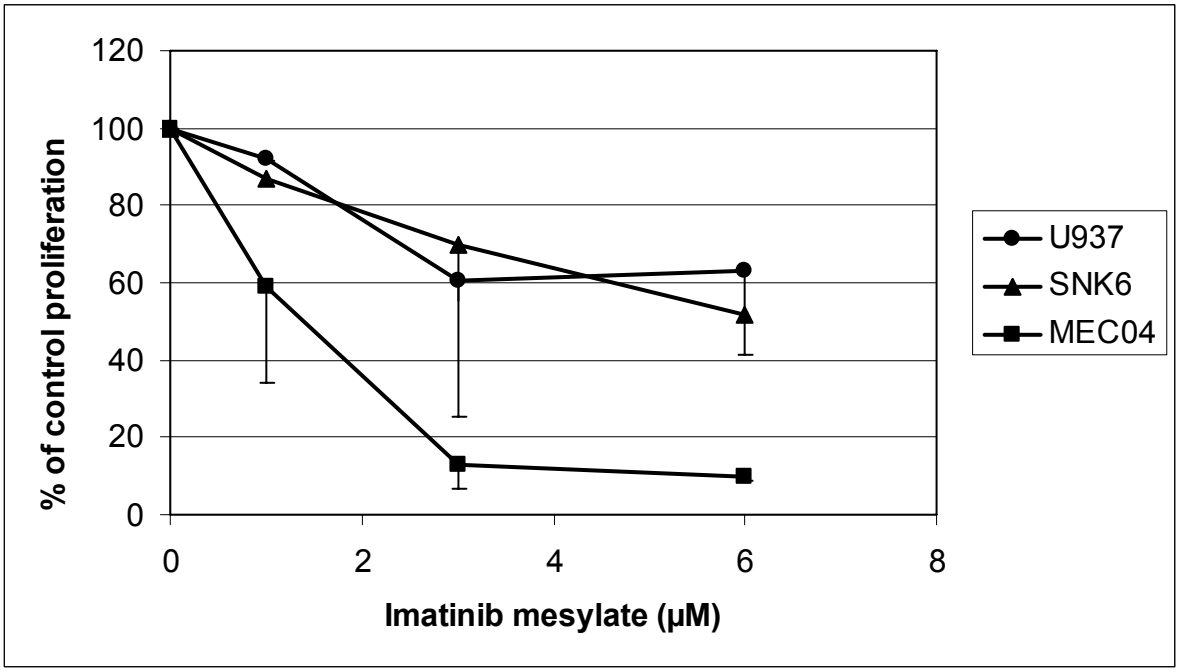
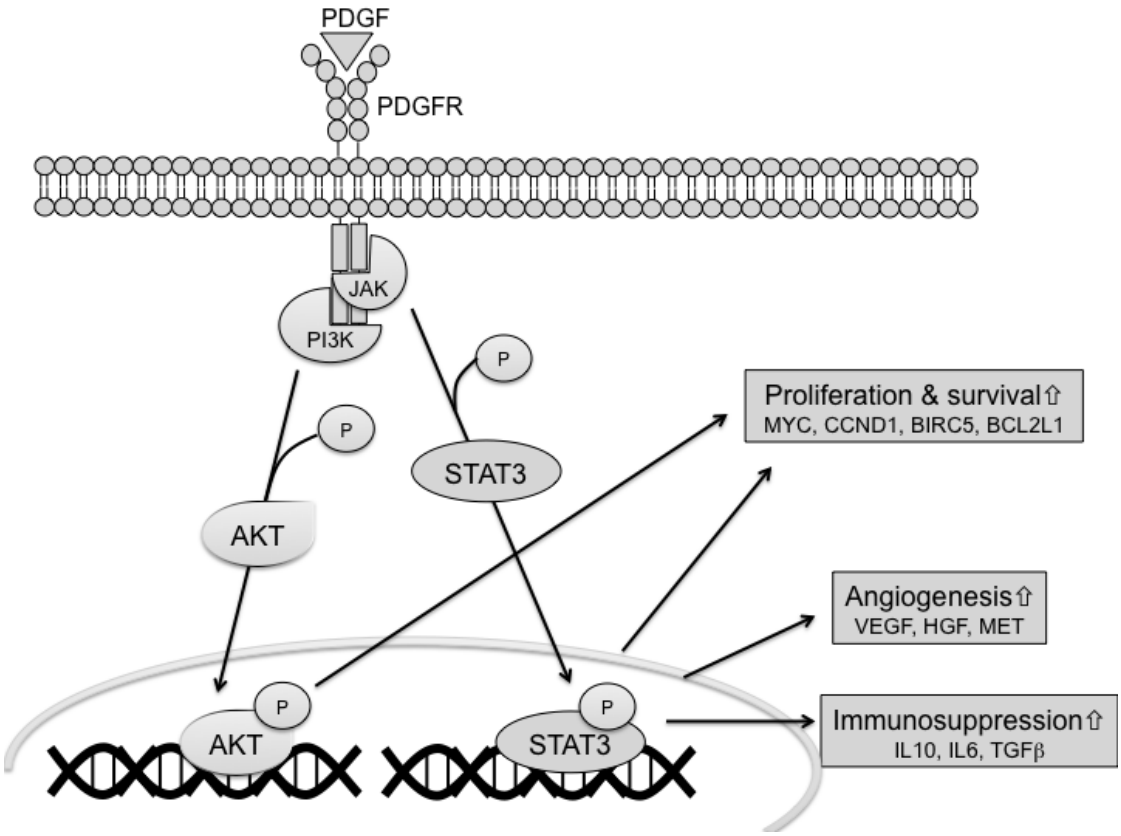

120
100
80
60
40
20
0
% of control proliferation
0
2
4
6
8
Imatinib mesylate (μM)
U937
SNK6
MEC04

Figure 7.


PDGF
PDGFR
JAK
PI3K
P
P
AKT
STAT3
Proliferation & survival⇧
MYC, CCND1, BIRC5, BCL2L1
Angiogenesis⇧
VEGF, HGF, MET
Immunosuppression⇧
IL10, IL6, TGFβ
P
AKT
P
STAT3