ALK Gene Mutation and ALK Protein Expression in Advanced Neuroblastoma and Potential Value in Risk Stratification in Fine Needle Aspiration Biopsy Samples

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Abstract

Objective: Anaplastic Lymphoma Kinase (*ALK*) gene gain-of-function point mutation leading to its overexpression has recently been identified and targeted in neuroblastoma (NB). We evaluated *ALK* gene mutation and its protein expression in cases of NB on fine needle aspiration biopsy (FNAB). **Material and Methods:** FNAB diagnosed cases of NB (n=56) were evaluated with cell blocks for *MYCN* amplification and ALK protein expression by Fluorescence in-situ hybridization and immunocytochemistry respectively. MGG stained smears (n=22) were used for Next generation sequencing (NGS) analysis using the Cancer Hotspot panel (version2) on Ion Torrent S5 platform. Staging and risk assignment as per International Neuroblastoma Risk Group (INRG) was performed and managed. All the parameters were correlated with overall survival. **Results:** ALK protein showed cytoplasmic expression in 65% of cases and did not correlate with *MYCN* amplification (p=0.35), INRG groups (p=0.52), and overall survival (p=0.2); however, ALK+ poorly differentiated NB showed better prognosis (p=0.02). ALK negativity was associated with poor outcome by Cox proportional hazard model (hazard ratio=2.36). *ALK* gene, exon 23 missense mutations (F1174L) were seen in 2/21 cases with an allele frequency of 8% and 54%. Both these cases showed ALK protein expression and died of disease within 1 and 17 months respectively. A novel IDH1 exon 4 mutation was also detected. **Conclusion:** *ALK* expression is a promising prognostic as well as a predictive marker in advanced NB along with traditional prognostic parameters. FNAB smears are suitable for NGS and *ALK* gene mutation confers a poor prognosis.

Introduction

Neuroblastoma is an embryonal tumor originating from the autonomic nervous system with a heterogeneous clinical and biological spectrum.[1] There is a wide range of clinical outcomes from spontaneous regression or maturation to relentless progression leading to death despite extensive multimodal therapies.[1-3] As per the International Neuroblastoma Risk Group (INRG) Task Force, age and stage at presentation, histologic category based on tumor differentiation and mitotic karyorrhectic index (MKI), DNA ploidy, *MYCN* amplification, and chromosome 11q status are parameters required for better risk stratification into very low, low, intermediate and high-risk groups.[4] Patients are managed according to their risk groups however the outcome for high-risk neuroblastoma patients remain poor, with overall 5-year survival below 50%.[4-5]

Anaplastic lymphoma kinase (*ALK*) gene and the corresponding protein ALK are important players in embryonic neural development and are highly expressed in that context.[6] The biological role of ALK in neuroblastoma has been studied in neuroblastoma cell lines.[7] The discovery of activating mutations in the tyrosine kinase domain of *ALK* as the major cause of hereditary neuroblastoma provides the first example of a pediatric cancer arising from germline mutations in an oncogene.[8] The role of MYCN in neuroblastoma is well established and evaluation of *MYCN* amplification in tissue samples is an important aspect of current risk stratification protocols including the INRG and COG algorithms for management.[4,9] Interestingly both

genes are just 13.2 MB apart on chromosome 2p. ALK, as a therapeutic target in primary neuroblastoma, represents a major research advance in this childhood cancer.[10] There have been a few studies on ALK protein expression in neuroblastoma by immunohistochemistry. [11-14] However, its utility as an independent prognostic marker and correlation to traditional prognostic markers such as age, tumor morphology, and *MYCN* amplification are still not well established and hence this was the main aim of this study. We also evaluated a subset of cases for *ALK* gene mutation by next generation sequencing to determine its frequency and have demonstrated its feasibility using fine needle aspiration biopsy samples.

Materials and Methods

Case selection

This was a mixed retrospective and prospective study of 60 cases of pre-therapy neuroblastoma diagnosed and managed at our hospital between January 2017 and December 2019. The study was conducted after Institute Ethics Committee approval vide letter no INT/IEC/2021/SPL-0. Archival cases of neuroblastoma were retrieved from the Departments of Cytology, Histopathology and Department of Paediatrics [Haematology-Oncology Division] of the Post Graduate Institute of Medical Education and Research Chandigarh, India.

Clinical data, Pathological Classification, and Risk Stratification and Follow-up:

The age, gender, symptoms, tumor size, location, imaging details wherever available, and treatment given (surgery / chemotherapy / radiation therapy / autologous stem cell transplantation) were noted from the records of Department of Paediatrics and the cohort characteristics are previously published.[15] Age, morphology, and MKI was combined to classify the cases into favorable and unfavorable histological subtypes as per INPC. The risk group stratification as per the International Neuroblastoma risk Group (INRG) was performed by accounting for the stage, age, morphology, MYCN, and ploidy. The information for 11q aberrations was not available and the INRG subtyping was done without it.

Management, Clinical follow-up and Outcome

The clinical management and follow-up information was available in a total of 50 patients. The cohort consisted predominantly of high-risk cases with varied management as feasible in a low income setting. A total of 14 patients received a complete protocol of high-risk treatment regimen (including induction chemotherapy, surgery, autologous stem cell transplantation, and radiation); 13 patients received chemotherapy followed by surgery, and 12 patients received only chemotherapy. Further in 4 patients debulking surgery was followed by adjuvant chemotherapy, only surgery was done in 2 patients and 1 patient underwent spontaneous remission without surgery. No treatment was given to 4 patients owing to the advanced stage of disease and/or complications due to disease per se. The duration of follow-up ranged from 7 days to 1200 days with a mean follow-up of 450 and a median of 480 days. Out of the 50 patients, 22 patients (44%) were alive without disease and 28 patients (56%) were dead at the end of follow-up period. Out of the 28 deceased patients, 10 suffered relapse post-treatment, 14 patients died due to progressive disease or complications related to therapy and 4 died without adequate therapy.

Fine Needle Aspiration, Cytomorphological and Mitotic-Karyorrhexis Index (MKI) evaluation

Fine needle aspiration cytology (FNAC) was performed by palpation/image-guidance from various sites and all smears and corresponding cell blocks with immunocytochemistry (if performed) were retrieved and re-evaluated by two expert cytopathologists (RS, MR). Cell blocks from FNA were prepared by plasma thromboplastin technique. These were categorized further as per previously published INPC criteria extended to cytopathology as undifferentiated, poorly differentiated, differentiating neuroblastoma or ganglioneuroblastoma.[16] Mitotic-karyorrhexis index (MKI) assessment was performed on well spread H&E-stained smears and their corresponding cell blocks using digital image visual assessment (DIVA).[17]

Immunocytochemistry (ICC):

In all cases with undifferentiated morphology, a suitable panel was applied for confirmation of neuroblastoma (positivity for neuronal markers like chromogranin, synaptophysin, and PHOX2B) and its differentiation from

other small round blue cell tumors (negativity for other markers like CD45, CD99/MIC2, Vimentin, desmin, myogenin). ICC for ALK protein was performed on cell blocks with adequate cellularity. The recommended staining protocol for VENTANA anti-ALK (D5F3 clone) CDx Assay and Rabbit Monoclonal Negative Control Ig with Opti View DAB IHC Detection Kit and Opti View Amplification Kit on a Benchmark XT instrument was followed. Positive control for immunocytochemistry was taken as section of appendix/colon with ganglion cells. ICC scoring was carried out using H-score wherein the tumor cell percentage positivity (in increments of 10) with 0, 1+, 2+ and 3+ intensity was evaluated. The percent positivity multiplied by the intensity was added up to obtain the final H-score as follows: H score= (0x % positive) + (1+x % positive) + (2+x % positive) + (3+x % positive). A cut-off of 100 was taken for statistical analysis with a score>100 being moderate to high ALK expression.

MYCN amplification by Fluorescence in situ hybridization (FISH):

FISH was performed either on smears (liquid-based preparation / destained MGG stained smear) or on cell block section using Vysis LSI N-MYC (2p24) spectrum green/ CEP 2 spectrum orange FISH probe (Abbott Laboratories, USA) and visualized by Olympus BX-51 FISH imaging system. A minimum of 100 nuclei were evaluated in all cases and > 10 *MYCN* signals were taken as positive for amplification. Ploidy was determined by counting the number of signals with the CEP probe.

Targeted Next generation sequencing for gene mutations

One representative and cellular FNA smear was photographed for documentation and the entire smear was scraped off the slides using a surgical blade manually. DNA was extracted using the Qiagen DNeasy Blood and Tissue Mini kit (Qiagen,Gmbh, Germany) and quantified by using Qubit ds DNA HS Assay kit (Thermo Fischer Scientific, Oregon). Only samples with a minimum DNA concentration of 2ng/µl were selected for NGS. An amplicon library was generated from 30-50ng/ µl of DNA from each sample using the Ion Ampliseq Cancer Hotspot Panel version 2.0 (Life Technologies and Ion AmpliSeq Library Kit 2.0 (Ion Torrent, USA) which evaluates hotspots in 50 genes including *ALK, IDH1, TP53* and others (supplementary table 1). Following PCR amplification of target sequences, barcodes were ligated to the amplicons using the Ion Xpress Barcode Adaptors Kit (Life Technologies). 100pM of DNA library of each sample was taken, pooled and run using Ion PGM Hi-Q OT2 kit. Emulsion PCR was performed manually using the Ion Xpress Template Kit (Life Technologies) followed by manual breaking and isolation of ion spheres (ISP). Selective ISPs with DNA were isolated and sequenced on an Ion 318 chip using Ion PGM Hi Q Sequencing kit and Ion S5 sequencer. Appropriate quality control parameters were assured with at least 300000 reads with a quality score of AQ20; minimum coverage of 250X and an allele frequency cut-off of 5%. The data files were analyzed by Torrent Suite software V2.0.1 (Life Technologies) using Hg19 as the reference; variants were detected using the Torrent Variant Caller software V1.0 (Life technologies) and visualized by Integrative Genomics viewer. Any variant observed was checked in the COSMIC and ClinVar databases and classified as per ACMG guidelines as benign, variant of uncertain significance (VUS), likely pathogenic and pathogenic.

Statistical analysis

RStudio version 1.3.1093 software was used for all statistical analyses. Chi-square ($\chi 2$) significance test was used to detect significant relationships between various parameters and prognostic factors. Kaplan-Meier curve was used to analyze overall survival among follow-up cases. A p value<0.05 was considered statistically significant. A multivariate COX regression hazard model was applied to evaluate the interplay of all the variables.

Results

Cohort Demographics

The clinical and pathological features of the cohort consisting of 60 patients of neuroblastoma are previously published.[15] Our cohort had older age, slight male preponderance, and a higher stage at presentation. The most common subtype was poorly differentiated neuroblastoma (55%, 33/60) followed by undifferentiated neuroblastoma (42%, 25/60) and ganglioneuroblastoma (3%, 2/60). There was no case of differentiating

neuroblastoma in this cohort. MKI could not be evaluated on smears in 10 cases due to poor smear quality, low cellularity and/or crushing artifact. In the remaining 50 cases, MKI was high in 39, intermediate in 4, and low in 7 cases. Prognostic evaluation by age combined with cytomorphological type and MKI was applied, and as per INPC, the majority had unfavorable cyto-histology (n=52, 87%) compared to favorable cyto-histology (n=8,13)

MYCN Amplification and International Neuroblastoma Risk Grouping [INRG]

A total of 46 cases were evaluated after excluding 4 cases (due to technical reasons); 48% (22/46) cases showed *MYCN* amplification and 52% (24/46) showed no amplification.

INRG risk stratification of the cohort was performed by combining age, stage, histologic category/grade, ploidy, and MYCN status. Majority were in the high-risk group (n=36, 60%) followed by intermediate-risk group (n=13, 22%) and low-risk group (n=11, 18%) respectively.

Anaplastic Lymphoma Kinase (ALK) protein expression by Immunohistochemistry

A total of 54 cases were evaluated for ALK protein expression after excluding 6 cases for technical reasons. ALK expression was mainly cytoplasmic and granular in nature (Fig.1), nuclear positivity was not seen. Some cases also showed intense membranous positivity and strong positivity in the neuropil. ALK expression was heterogeneous in 17 out of 54 (32%) cases. H-scoring was performed as described in the methods and overall, 35 cases (65%) with an H-score of [?]100 showed moderate to strong ALK positivity, whereas 19 cases (35%) with <100 H-score were negative (Fig.1).

Targeted Next generation sequencing for *ALK* gene and other gene mutations

The amount of DNA extracted ranged from 9.9-39ng/µl. Only 2 (out of 21) cases showed *ALK* gene, exon 23 missense mutations on locus Chr2:29443697 (COSM28057), transcript NM_004304.5, c.3520T>C, and on locus chr2: 29443695, transcript NM_004304.5, c.3522C>G, both resulting in amino acid change p.Phe1174Leu (F1174L) with allele frequency (AF) of 8.3% and 54% respectively. Both showed ALK protein expression. They were aged 9 and 60 months, showed poorly differentiated and undifferentiated morphology respectively, with high Mitoses-Karyorrhexis index,*MYCN* amplification, unfavorable histology as per INPC and died of disease within 1 and 17 months respectively. One case is depicted in Fig.2.

A novel *IDH1* (isocitrate dehydrogenase1) exon 4 missense mutation in locus chr2: 209113151, transcript NM_005896.4,c.356G>A, amino acid change p.Arg119Gln (R119Q) was seen with 46.4% AF in an 18-month-old female with undifferentiated morphology, *MYCN* amplification but no ALK protein expression depicted in Fig.3 and is alive without disease at 16 months follow-up.

Correlation of ALK expression with traditional prognostic parameters

The overall survival correlated with patient's age (p=0.03), stage (p=0.01), morphology (p=0.036) and INRG risk groups (p=0.0001) as previously reported.[15] There was no correlation of ALK protein expression with INRG subgroups although higher levels were observed in the high-risk group (Fig.4A). Univariate analysis revealed ALK protein expression did not significantly correlate with the outcome (Fig.4B); furthermore, the combination of ALK protein expression with*MYCN* amplification status also revealed no association with the outcome (Fig.4C). In ALK positive cases (Table 1),*MYCN* amplification revealed no association with outcome (Fig.4C). Hence, we analyzed the effect of morphology on outcome within the group of ALK positive cases which revealed that ALK positive poorly differentiated neuroblastomas had better survival (p=0.02) than the other subgroups which had almost similar overall survival (Fig.4D).

Multivariate COX regression hazard model

A model was proposed to calculate the Cox Proportional Hazard Ratios by utilizing 4 factors- age, cytomorphology, stage, and ALK protein expression in 39 cases and data is depicted as a forest plot (Supplementary Fig 1). The global p-value using Log Rank test was significant (p= 0.04). Based on the hazard ratios, ALK negativity was associated with poor outcome.

Discussion

Neuroblastic tumors are subjected to risk stratification for management purposes and have an overall poor prognosis despite the extensive multimodal therapies highlighting the need for discovery of alternative therapeutic targets. [4,5,18] One such target is the Anaplastic Lymphoma Kinase or *ALK* gene which has been the subject of intense research in neuroblastomas and reviewed by several authors recently[7,19] This study was a small step to establish the expression of ALK protein in samples obtained by fine needle aspiration biopsy and evaluate its role in prognostication and risk stratification. We further evaluated ALK gene mutations in a subset of cases by NGS on cell scrapes.

ALK protein is a receptor tyrosine kinase that has an expression restricted to the developing central and peripheral nervous system and is critical for embryonal neuronogenesis in the sympathetic ganglia and tumors showing ALK overexpression referred to as ALKomas might benefit from directed therapies.[20] ALK protein expression in tissues may be evaluated by immunohistochemistry. There are many antibodies commercially available and a comparative analysis of various antibody clones revealed the high sensitivity of D5F3 clone, justifying its choice in this study.[13] In this study, ALK expression was evaluated on adequately cellular cell blocks from FNA samples and 65% (35/54) cases showed positivity. The heterogeneity of ALK expression in most cases necessitated H-scoring which allowed the selection of strongly positive cases even if heterogenous and excluded the weak positives. There are a few studies that have evaluated ALK protein in histopathology sections including tissue microarrays with a frequency of 41-66% which is in agreement with our observations. [11,13,14] Our results on FNA cell blocks are comparable to studies on conventional histopathology specimens and thus it may be concluded that cell blocks are an acceptable substitute for evaluating ALK expression by immunocytochemistry.

ALK gene mutations in neuroblastoma is well studied.[19,21,22] There is however no report from India and from cytology samples. Tumour DNA was extracted from MGG stained representative and cellular smears, a novel aspect of the study. The use of smears for molecular studies including NGS has been highlighted previously.[23] Out of 21 cases evaluated, 3 pathogenic/likely pathogenic mutations were observed, two in *ALK*gene and one in the *IDH1* gene. Both *ALK* mutations resulted in F1174L, which is the most frequent oncogenic driver mutation seen in 12.4% cases of sporadic neuroblastomas.[8,24] Bresler et al in their comprehensive genomic study of *ALK* mutations in 1596 samples revealed tyrosine kinase domain mutations in 8% cases involving 3 hotspots R1275Q, F1174 and F1245 accounting for 43%, 30% and 12% of cases respectively.[21] The F1174 was associated with an aggressive tumor phenotype as was observed in our cases too.

An interesting aspect of neuroblastoma is the chromosome 2p, which is home to two important oncogenes, *MYCN* at 2p24 and *ALK* at 2p23.2. In our study, *MYCN* amplification by FISH did not correlate with ALK protein expression (p=0.35). However, immunohistochemistry for MYCN protein was significantly associated with ALK protein expression.[14] A meta-analysis of neuroblastomas comparing mutations in the *ALK* gene mutation spectrum with *MYCN* amplification status found *ALK*mutations occur in equal frequencies across all genomic subtypes, but the F1174L mutants were observed in a higher frequency in*MYCN* -amplified tumors, demonstrated higher transforming capacity resulting in more aggressive tumors.[25] Interestingly the *ALK* ^{F1178L} knock-in mice (corresponding to F1174L in humans) resulted in enhanced proliferation and extended neurogenesis but no tumor formation in the absence of*MYCN* co-expression.[26] In another study on a mouse model, a co-operative interaction of$ALK^{F1174L}/MYCN$ led to aggressive and lethal tumors with activation of the PI3K/AKT/mTOR and MAPK pathways.[27]

Out of the 50 patients with complete follow-up in this study, 28 patients (56%) died due to disease progression and 22 patients (44%) were alive without disease. In spite of this, in this small cohort of 60 patients, INRG stratification into high, intermediate, and low-risk groups had a significant effect on outcome (p=0.02) and overall survival (p=0.0001), similar to previous reports.[28,29] ALK expression did not correlate with the age of patient, morphological type, stage (stage 4 vs non-stage 4), *MYCN* status, and INRG group. We chose a cut-off of an H-score of 100 for statistical analysis. Those less than 100 represented negative or with low levels of expression. ALK protein expression independently had no correlation with outcome and overall

survival which is in concordance with a previous report by Lee et al[11] and De Brouwer et al[25]. Hence, we evaluated the effect of various parameters in the ALK positive cases wherein morphology correlated well with outcome (p=0.01) and overall survival (p=0.02) with poorly differentiated having a better overall survival than undifferentiated neuroblastoma. Our pilot observations need confirmation in larger study cohorts. When risk stratification was applied only to ALK positive tumors, significant results towards overall survival were observed (p=0.03). Weiser et al found a significantly higher number of*ALK* mutations in high-risk versus low-risk cases (p=0.01) but there were no significant results with age or histology similar to our study.[30] On the other hand, Carpenter et al reported a correlation of increased *ALK* mRNA with poor prognosis.[19] In vitro studies of ALK signaling in cultured neuroblasts resulted in transient proliferation followed by differentiation and long-term survival of post-mitotic neurons.[26] A novel *IDH1* gene missense mutation was observed in a single case. *IDH1* gene mutations are common in gliomas and other tumors of the CNS and hemato-lymphoid malignancies.[31] Functional in vitro studies on cell lines or mouse models are required to elucidate its role in neuroblastoma.

In view of the complex interplay of so many variables for risk stratification in neuroblastoma, all parameters which significantly correlated with outcome such as age, stage, cytomorphology were evaluated along with ALK expression in a multivariate Cox proportional hazard model which gave significant results (p= 0.04). A similar analysis substituting ALK with *MYCN* was performed which was not statistically significant. Our pilot study data suggests that while ALK is not an independent prognostic factor, that it may have a role along with other traditional prognostic factors with ALK negativity predicting a worse outcome (Hazard Ratio, 2.3). A subset of poorly differentiated neuroblastomas who are ALK positive have a good survival and would otherwise would have been categorized as having a poor prognosis based on traditional parameters. Our data is limited due to the small cohort size and a potential inclusion bias of advanced cases which are subjected to FNAB. However, these observations need validation on studies with larger number of cases with better statistical power.

On the other hand, ALK has emerged as a potential predictive therapeutic biomarker and an ideal candidate for targeted therapy in neuroblastoma due to the dismal prognostic scenario.[32] Several ALK inhibitors are in various phases of drug development and scrutiny in neuroblastoma and have shown promising results. Apart from crizotinib, other ALK inhibitors like lorlatinib, brigatinib, ceritinib, alectinib, entrectinib are undergoing clinical trials along with a combination of TRKA/B/C, MDM2, and CDK4/6 inhibitors and these are reviewed by Pacenta and Macy.[33] These trials offer a ray of hope in neuroblastoma, a tumor that remains a challenge for the pediatric oncologist. Hence detection of ALK protein expression levels in any sample of neuroblastoma assumes significance.

Conclusion

ALK protein expression evaluated on FNA cell blocks was found to be useful in risk stratification in addition to the traditional parameters of age, stage (INSS), morphology (INPC), and risk group (INRG). Cell scrapes are suitable for NGS and the F1174L *ALK* gene mutation confers a poor prognosis in neuroblastoma.

Conflict of Interest Statement All authors hereby declare that they have No conflict of interests.

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Figure Legends

Figure 1. ALK protein immunocytochemistry on cell block sections of neuroblastoma.

A. Completely negative; B. 1+ granular cytoplasmic positivity; C. 2+ granular cytoplasmic positivity, D. 3+ strong diffuse ALK positivity which is cytoplasmic as well as membranous, E. Granular positivity seen inside the neuropil [Immunoperoxidase; o.m – A-Ex200], F. Dot plot of ALK H-scoring done in ascending value. Each dot represents a case. The dotted line marks the cut-off value of 100.

Figure 2. *ALK* gene mutated undifferentiated neuroblastoma in a 10-month-old. A. Cellular FNA smears with undifferentiated small blue round cells with high mitosis and karyorrhexis; B. Cell block immunocytochemistry showing ALK protein expression; C, FISH showing *MYCN* amplification; D, Screenshot from Integrated genomics viewer showing *ALK* gene mutation on locus chr2: 29443695 resulting in F1174L [A, May-Grünwald-Giemsa stain; B, immunoperoxidase stainx200; C, FISH Vysis LSI N-MYC (2p24) spectrum green/ CEP 2 spectrum orange FISH probe with DAPI counterstain; A x400, B x400, C x1000 o.m.]**Figure 3.** *IDH1* gene mutated poorly differentiated neuroblastoma. A-B. Cellular smear and corresponding cell block showing rosetting of neuroblastic cells with abundant neuropil in the background; C. Cell block immunocytochemistry showing no ALK protein expression; D. FISH showing non-amplified *MYCN;* E. Screenshot from Integrated genomics viewer showing *IDH1* exon 4 missense pathogenic mutation on locus chr2: 209113151 resulting in amino acid change R119Q. [A, May-Grunwald-Giemsa stain; B, Hematoxylin-Eosin stain; C, immunoperoxidase stainx200; D, FISH Vysis LSI N-MYC (2p24) spectrum green/ CEP 2 spectrum orange FISH probe with DAPI counterstain; A-C x200, D x1000 o.m.]

Figure 4. A. ALK protein expression in the three INRG subgroups. B-D. Kaplan-Meier curve showing relationship of overall survival in neuroblastoma with ALK (positive versus negative cases), combination of ALK and *MYCN* (4 subgroups) and combination of ALK and morphology (4 subgroups). Tick marks indicate censored individuals.

Supplementary Fig. 1. Cox Proportional Hazard Model and Forest plot showing Hazard ratios (HR) with 95% confidence interval.

Table 1 . Association between morphology and *MYCN* status parameters in ALK positive cases

Neuroblastoma Cytomorphology	***MYCN* Amplified**	***MYCN* Not Amplified**	**Total (n=28)**	**P value**
Undifferentiated	11	3	14	**0.0079**
Poorly differentiated	4	10	14	

Author Contribution Statement:

Neha Bhardwaj: Study conceptualization, data curation (clinical and pathology), formal analysis, statistics,

manuscript original draft, editing and final draft.

Manish Rohilla: Data curation (cytopathology), analysis, manuscript editing and final draft.

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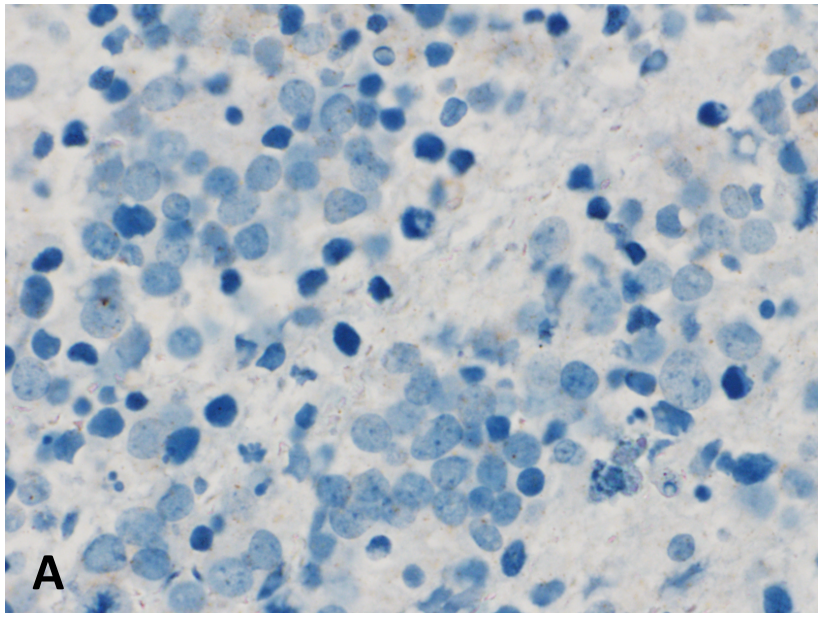
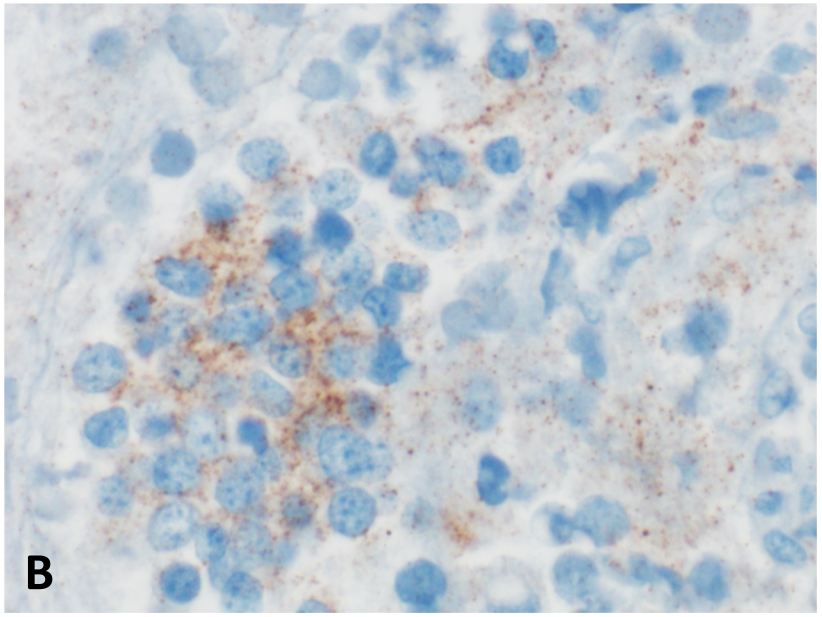
Deepak Bansal: Clinical data curation, analysis, manuscript editing and final draft.

Nandita Kakkar: Data curation (histopathology), manuscript editing and final draft

Radhika Srinivasan: Study conceptualization, data curation (pathology), analysis, statistics, manuscript editing and final draft.


A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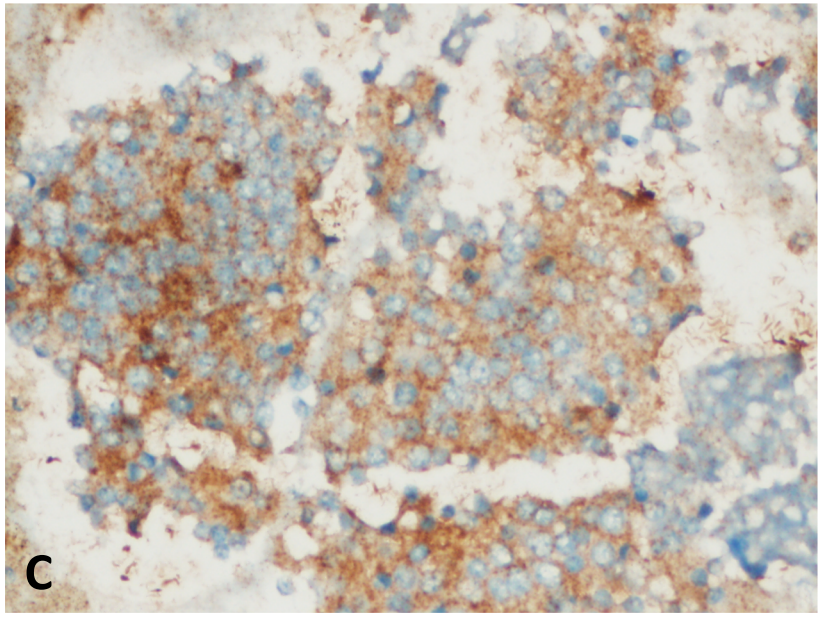
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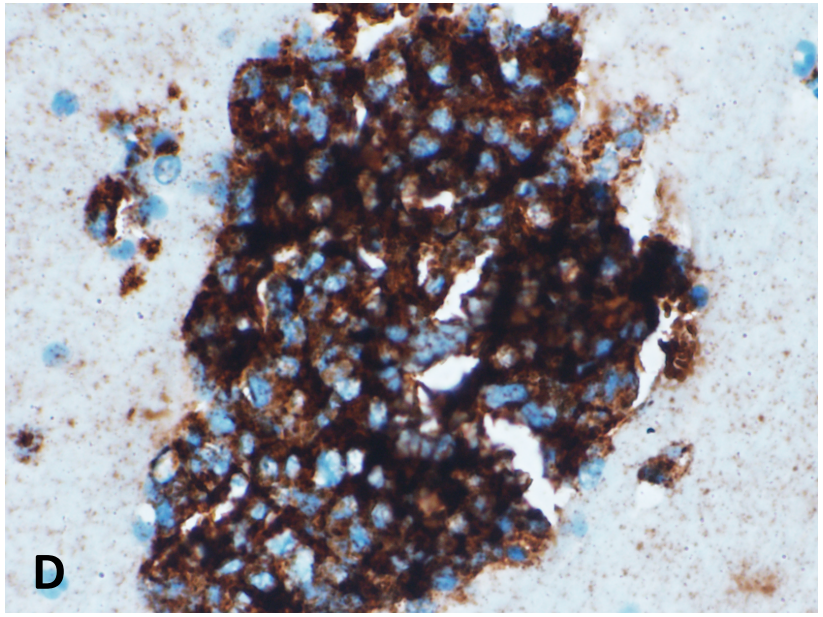
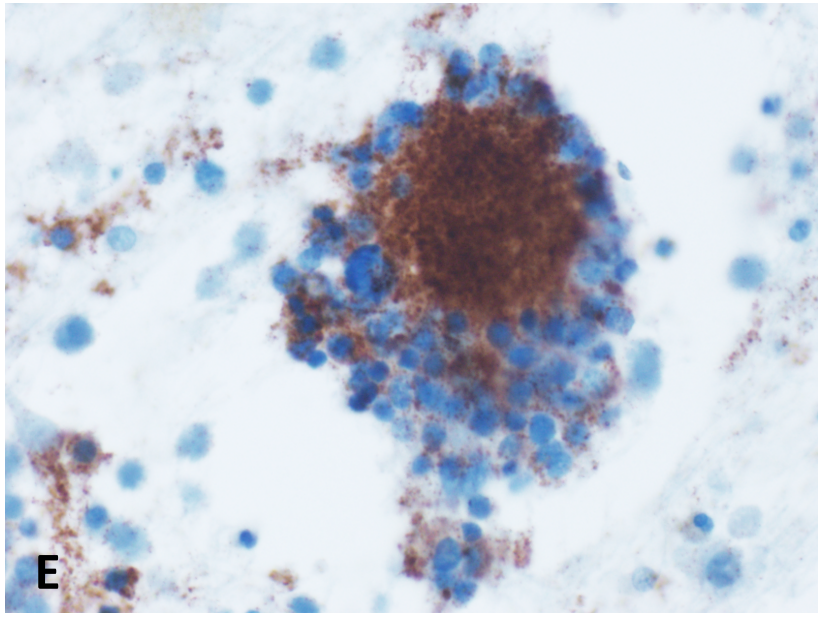

D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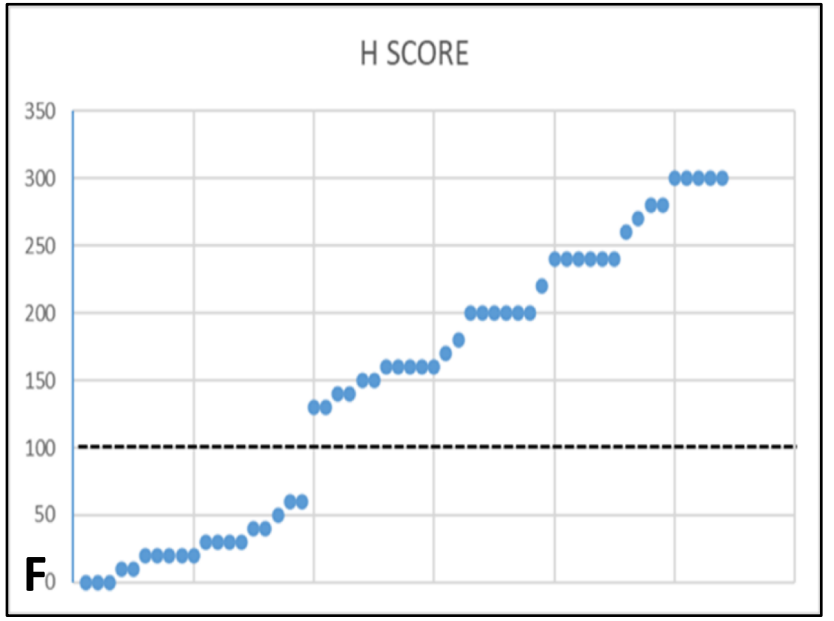
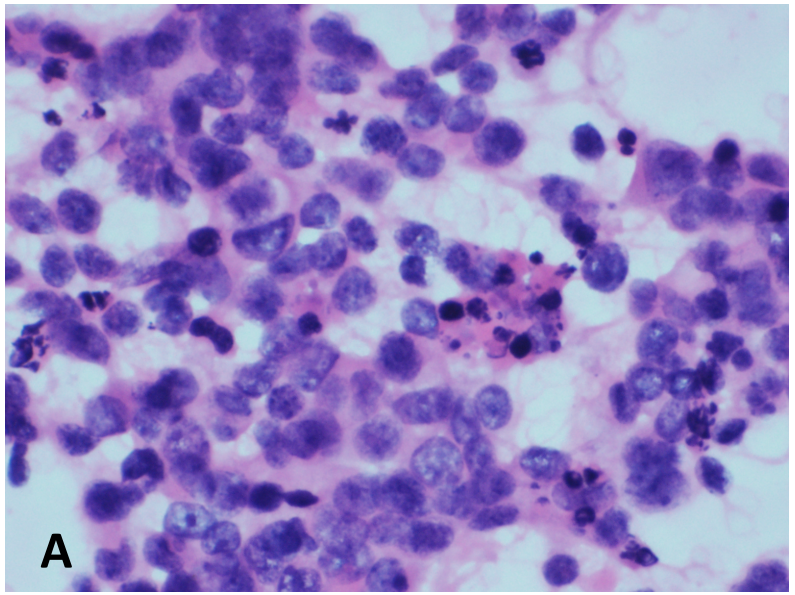

H SCORE
350
300
250
200
150
100
50
0
F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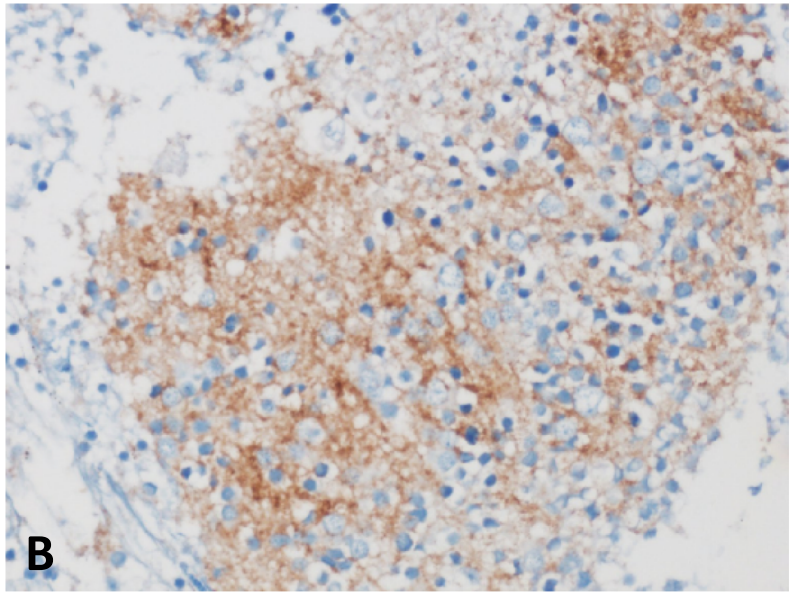
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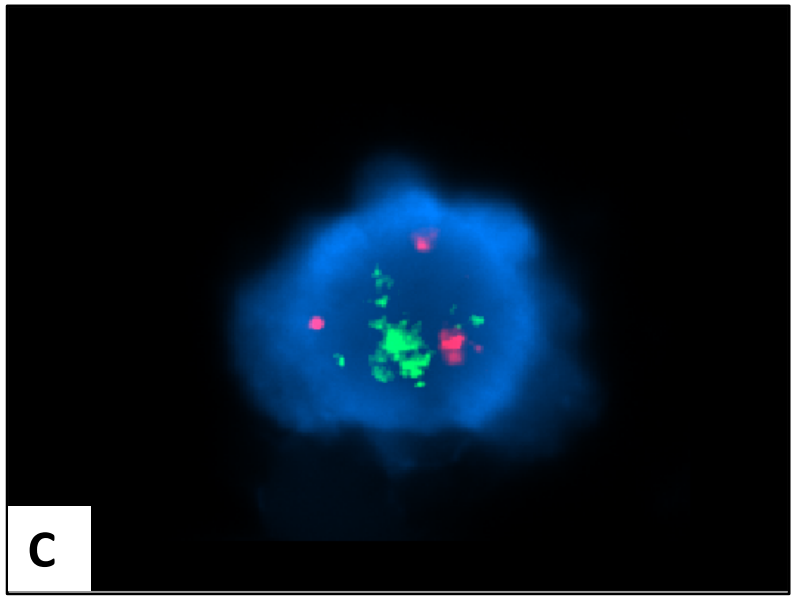
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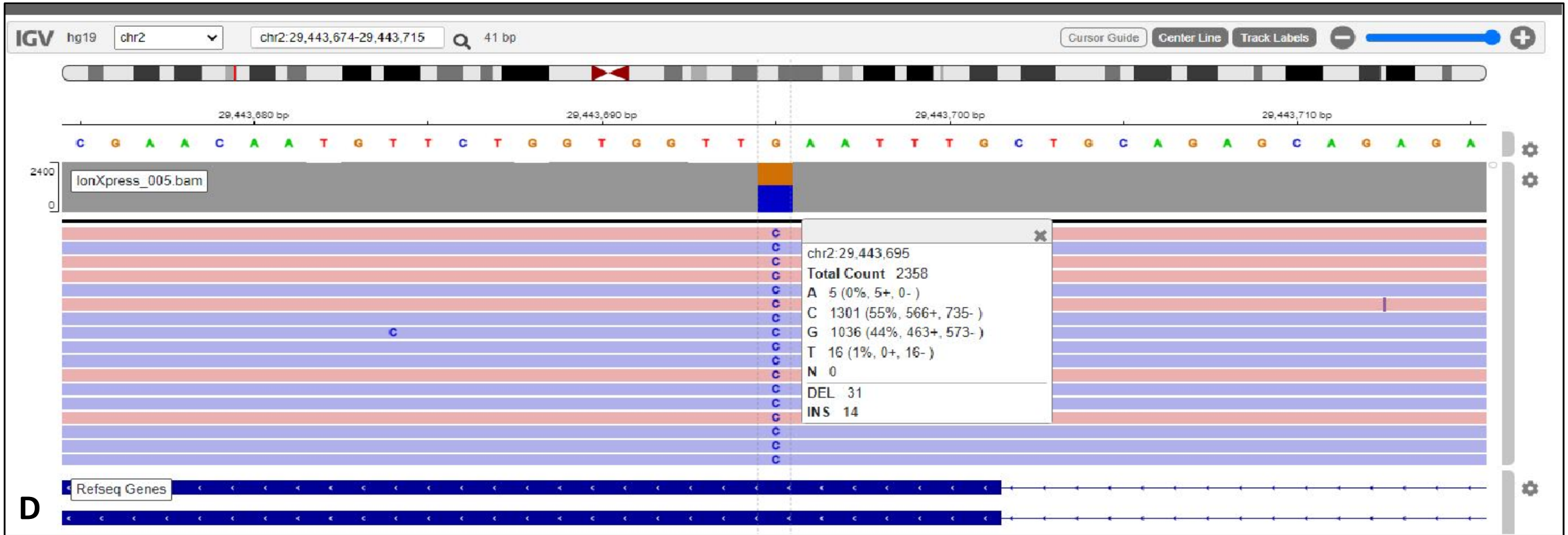
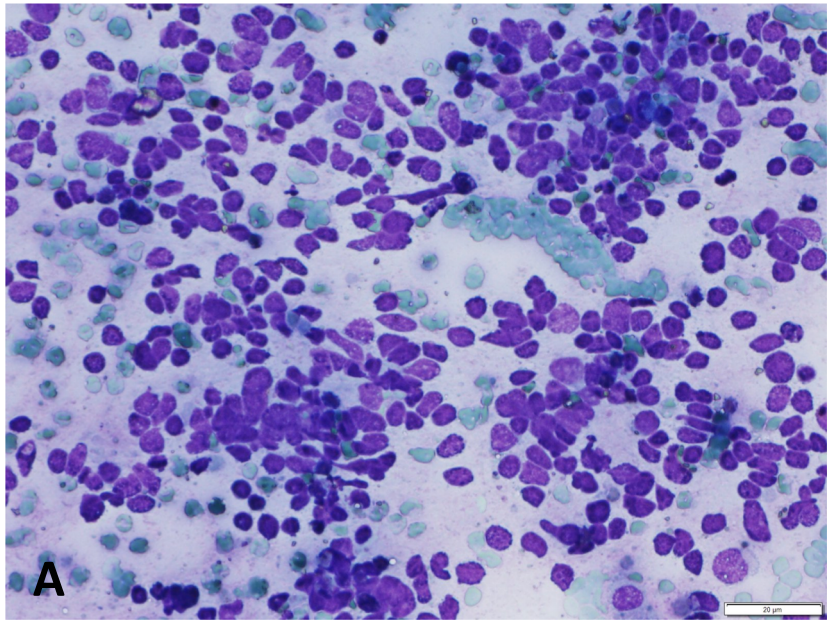
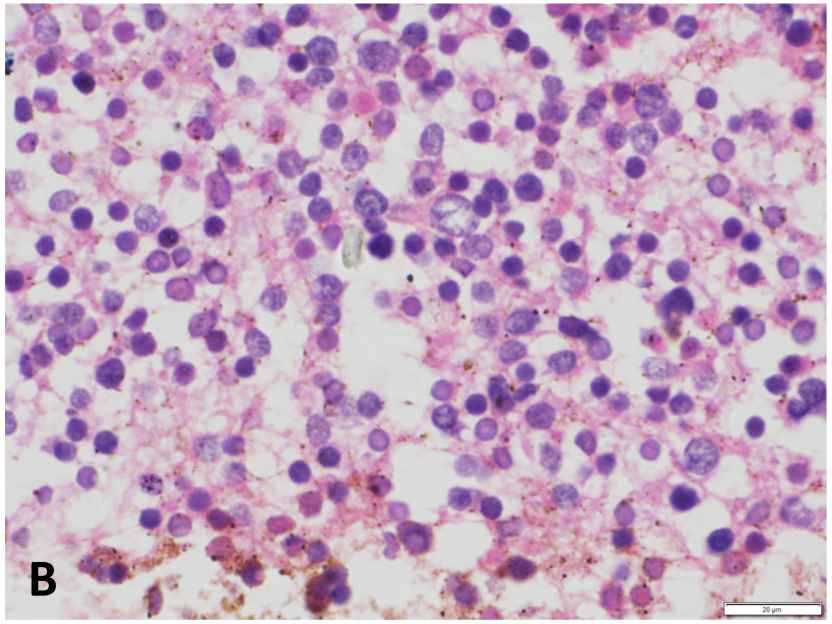
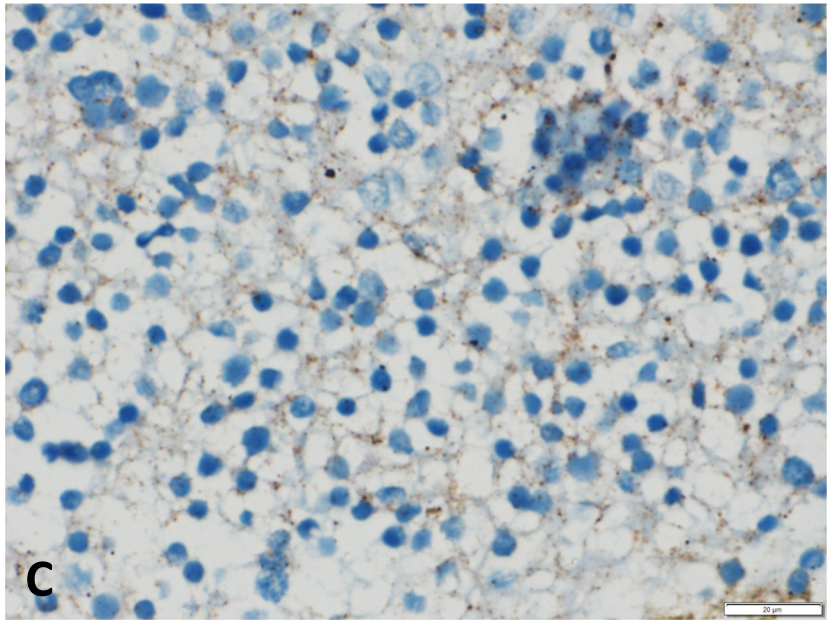

IGV
hg19
chr2
chr2:29,443,674-29,443,715
41 bp
Cursor Guide
Center Line
Track Labels
29,443,680 bp
29,443,690 bp
29,443,700 bp
29,443,710 bp
C G A A C A A T G T T C T G G T G G T T G A A T T T G C T G C A G A G C A G A G A
2400
0
IonXpress_005.bam
chr2:29,443,695
Total Count 2358
A 5 (0%, 5+, 0-)
C 1301 (55%, 566+, 735-)
G 1036 (44%, 463+, 573-)
T 16 (1%, 0+, 16-)
N 0
DEL 31
INS 14
Refseq Genes
D


A


B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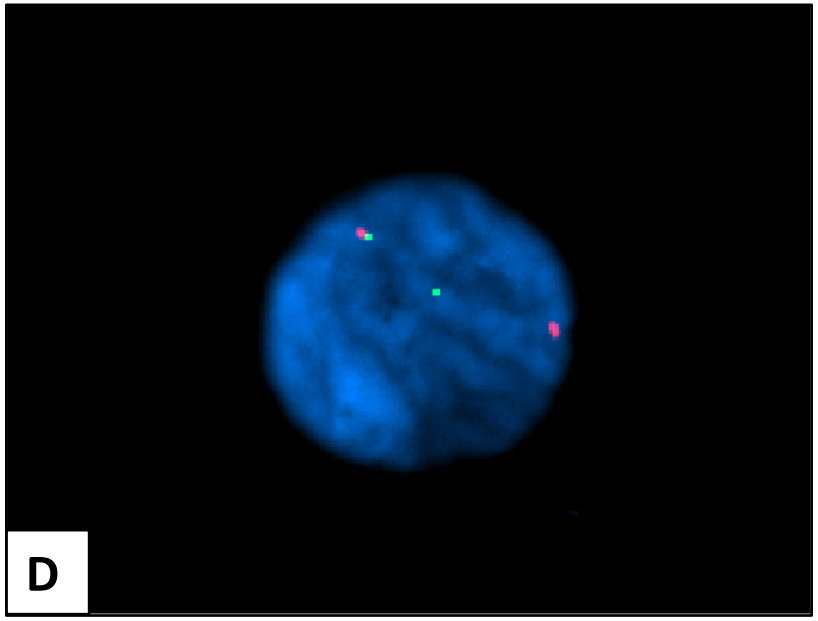
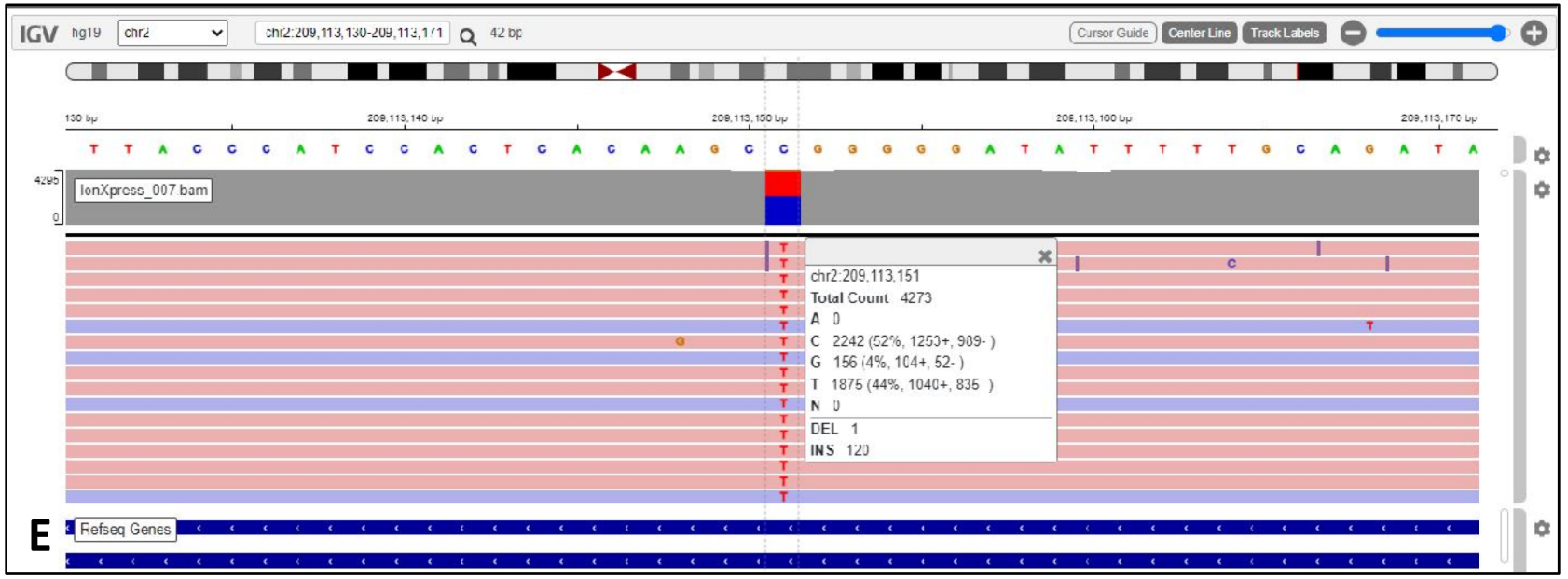
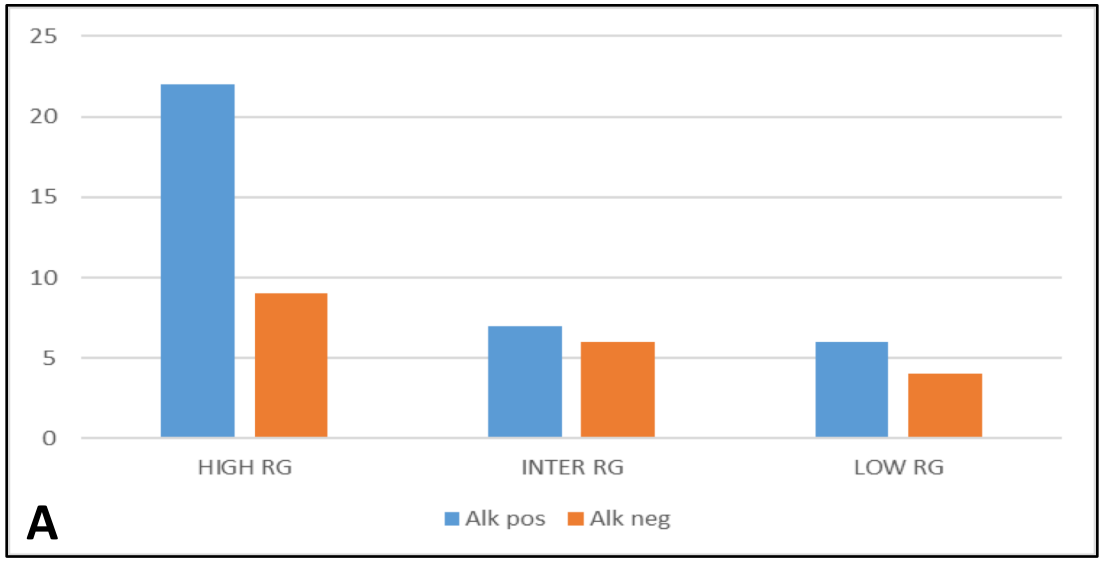
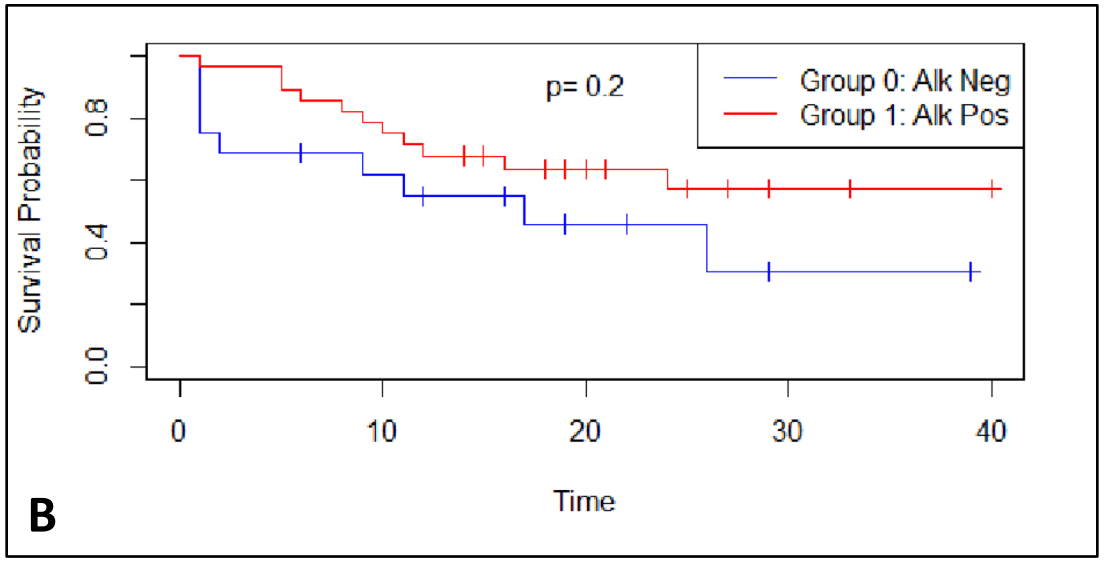
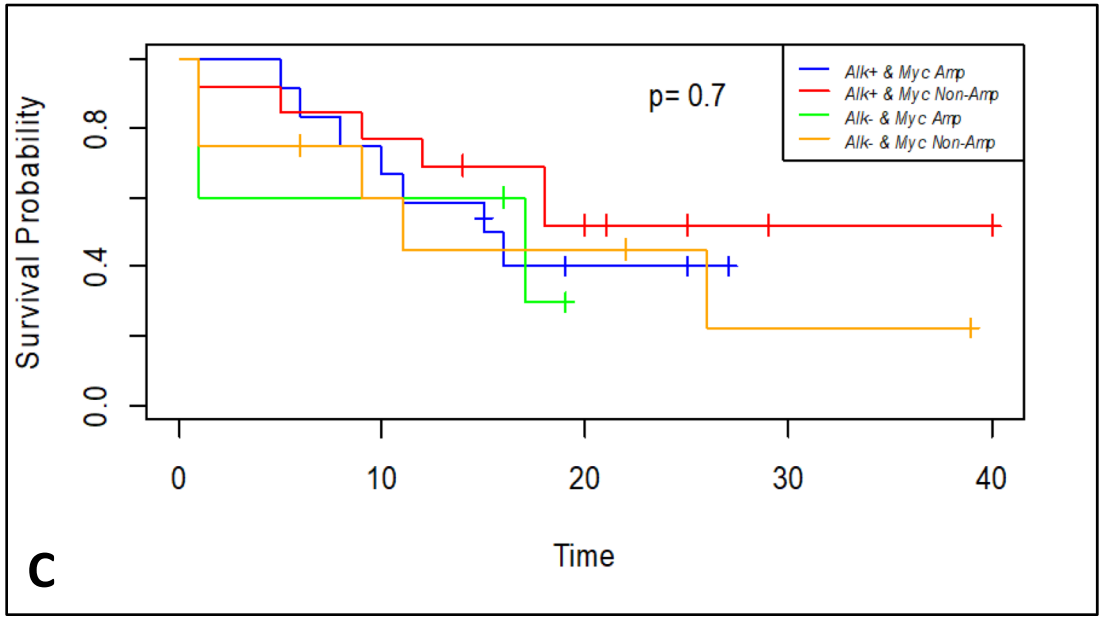
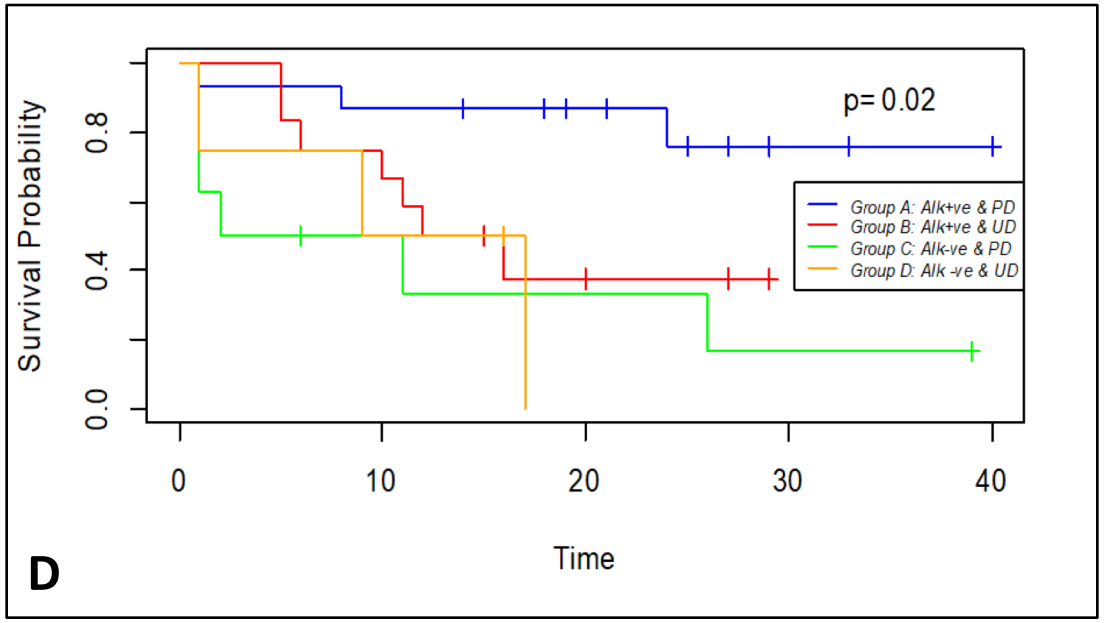

D


IGV
hg19
chr2
chr2:209,113,130-209,113,171
42 bp
Cursor Guide
Center Line
Track Labels
130 bp
209,113,140 bp
209,113,150 bp
209,113,160 bp
209,113,170 bp
IonXpress_007.bam
chr2:209,113,151
Total Count 4273
A 0
C 2242 (52%, 1253+, 989-)
G 156 (4%, 104+, 52-)
T 1875 (44%, 1040+, 835)
N 0
DEL 1
INS 120
Refseq Genes
E


25
20
15
10
5
0
HIGH RG
INTER RG
LOW RG
Alk pos
Alk neg
A


p= 0.2
Group 0: Alk Neg
Group 1: Alk Pos
Survival Probability
0.0
0.4
0.8
0
10
20
30
40
Time
B


p= 0.7
Alk+ & Myc Amp
Alk+ & Myc Non-Amp
Alk- & Myc Amp
Alk- & Myc Non-Amp
Survival Probability
0.0
0.4
0.8
0
10
20
30
40
Time
C


p= 0.02
Group A: Alk+ve & PD
Group B: Alk+ve & UD
Group C: Alk-ve & PD
Group D: Alk -ve & UD
Survival Probability
0.0
0.4
0.8
0
10
20
30
40
Time
D