ORIGINAL ARTICLE

Molecular Biomarkers Detected Using Fluorescence in situ Hybridization in a Filipino with Retinoblastoma

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ABSTRACT

Background and Objective. Retinoblastoma is one of the most common intraocular cancers among children usually caused by the loss of retinoblastoma protein function. Despite being a highly heritable disease, conventional diagnostic and prognostic methods depend on clinical examination, with limited consideration of cancer genetics in the standard of care. *CD133*, *KRT19*, and *MUC1* are commonly explored genes for their utility in liquid biopsies of cancer including lung adenocarcinoma. To date, there are few extensive molecular studies on retinoblastoma in Filipino patients. To this end, the study aimed to describe the copy number of *CD133*, *KRT19*, and *MUC1* in retinoblastoma samples from a Filipino patient and quantitate the respective expression level of these genes.

Methods. Hematoxylin & Eosin (H&E) staining was utilized to characterize the retinoblastoma tissue while fluorescence in situ hybridization (FISH) using probes specific to *CD133*, *KRT19*, and *MUC1* was performed to determine the copy number of genes in retinoblastoma samples from a Filipino patient (n = 1). The gene expression of *CD133*, *MUC1*, and *KRT19* was quantitated using RT-qPCR.

Results. The H&E staining in the retinoblastoma tissue shows poorly differentiated cells with prominent basophilic nuclei. *CD133* was approximately 1.5-fold overexpressed in the retinoblastoma tissue with respect to the normal tissue, while MUC1 and KRT19 are only slightly expressed. Multiple intense signals of each probe were localized in the same nuclear areas throughout the retinoblastoma tissue, with high background noise.

Conclusion. These findings suggest that *CD133* is a potential biomarker for the staging and diagnosis of retinoblastoma in Filipino cancer patients. However, further optimization of the hybridization procedures is recommended.

Keywords: retinoblastoma, biomarkers, hybridization in situ, CD133

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INTRODUCTION

Retinoblastoma (RB) is the most common intraocular tumor in children below 15 years of age, accounting for about 2% of all cancers diagnosed annually in the US and Europe.[1,2] In the Philippines, there has been a reported fivefold increase in the incidence of retinoblastoma over the last four decades from 48/100,000 to 237/100,000 cases.[3] Clinical manifestations include leukocoria, a white reflection evident in the pupil, and the characteristic strabismus, which is the misalignment of the eyes observed when focusing on one object. Standard retinoblastoma diagnosis involves examining the intraocular fundus under general anesthesia

and ultrasound supplementation. In some cases, the use of computed tomography and magnetic resonance imaging is required. Several retinoblastoma treatments are available, including chemoreduction using vincristine, etoposide, and carboplatin, focal therapy, external beam monotherapy, and enucleation. However, retinoblastoma has a tendency to spread to the brain and bone marrow, but seldom to the lungs. The disease prognosis is affected negatively by extraocular extension and invasion along the optic nerve and choroid.[4]

Approximately 50% of retinoblastoma cases occur in individuals who have inherited a germline mutation of the RB allele, leading to a potential loss of control in the cell cycle and genomic instability.[5] The pathophysiology of retinoblastoma usually occurs as a result of the loss of function of the two alleles of retinoblastoma (RB1) in the chromosome 13q14 locus. *RB1* is a 190 kb gene with 27 exons that encodes for the 928-amino acid retinoblastoma protein (pRB), a cell cycle regulator.[5] During the G_0 and early G_1 phases, dephosphorylated pRB binds to the E2F transcription factor, blocking its transactivation domain. Hyperphosphorylation by cyclin-dependent kinases (CDKs) causes pRB to release E2F, resulting in the transcription of genes necessary to initiate the S phase of the cell cycle. In the Philippines, a study characterized retinoblastoma mutations in tumor samples obtained from Filipino patients. Among the six patients, four were observed to have no mutations in the exons, while one had no exons amplified. A novel missense mutation (R1861S) was identified in the cohort.[6]

Previous studies on retinoblastoma primary tumors and cell lines have found the presence of a few characteristics associated with cancer stem cells (CSCs). It was reported that RB tumor populations derived from both mice and human cell lines express adenine triphosphate-binding cassette transporter G2 (ABCG2) protein,[7] a cell surface marker capable of providing chemoresistance. This ability to survive chemotherapeutic treatment is believed to be attributed to the presence of CSCs.[8] In addition, other stem cell markers such as Octamer-binding transcription factor 4 (OCT4), Nestin, CD44, sex-determining factor Y-box 2 (SOX2), and paired box 6 (PAX6) were also identified to determine the tumorigenicity and origin of retinoblastomas.[9] The detection of these markers plays a vital role in characterizing the stemness of the Rb, which aids in elucidating their cellular behavior. In turn, this creates wider opportunities for proper intervention and targeted therapy.

Various studies related to RB have found CD133 to be expressed in heterogenous RB tumors and cell lines, suggesting its specificity to CSCs. CD133 is a transmembrane protein that is encoded by a single-copy gene on chromosome 4 (4p15.33).[10] Its expression in undifferentiated progenitor retinal cells increased the capacity for tumor growth and invasiveness, suggesting its possible use as a biomarker for the development of retinoblastoma.[11] Meanwhile, CD133 has been investigated in other malignancies elucidating its viability to be an excellent cancer biomarker. Studies on a pancreatic cancer cell line have revealed a positive correlation between the expression of CD133 and an increase in the induction of the epithelial-mesenchymal-transition (EMT) in cancer cells, resulting in increased tumorigenicity, invasiveness, and metastasis.[12] Further, it has been found that CD133 modulates the expression of HIF-1a in hypoxic conditions that promote EMT and cancer cell migration *in vitro*.[13]

Aside from CD133, other biomarkers such as mucin-1 (MUC1) and keratin 19 (KRT19) have been utilized to characterize CSCs in various malignancies. MUC1 is a transmembrane protein belonging to the mucin family coded in the long arm of human chromosome 1.[14] MUC1 is normally expressed in mammary epithelial cells, and has been considered a valuable gene marker in the diagnosis and detection of gastric cancer, breast cancer, and malignant mesothelioma.[15] Overexpression has been linked to the development of cancer and metastatic aggressiveness of cancer cells.[13] Conversely, keratin 19 (KRT19) is an acidic type 1 keratin specifically expressed in the periderm and human epithelial cells, encoded in chromosome 17q21.2. It is a marker of epidermal stem cells.[16] It has been identified as a tumor detector gene and a biomarker for breast cancer.[17] The Cypfra 21-1 fragment is a potential serum biomarker for thyroid carcinoma and hepatocarcinoma.[18] KRT19 modulates cancer stem cell reprogramming in breast cancer models.[19] Amplifications of KRT19 were significantly associated with lymph node-positive breast cancer and were found to be highly expressed in patients with pancreatic tumors, associated with larger tumor sizes, poorly differentiated tumors, and lymph node metastasis.[20,21]

As of present, there are few extensive molecular studies on retinoblastoma in Filipino patients.[6,22] Despite being a highly heritable disease, the standard of care for retinoblastoma does not take into consideration the genetics of cancer. To bridge the gap between molecular cytogenetics and clinical retinoblastoma, we observed the changes in the DNA copy number and gene expression of certain cancer stem cell genes, CD133, KRT19, and MUC1. Current methods for detecting and quantitating these cancer biomarkers include colorimetric assays, polymerase chain reactions, electrophoresis, optical methods, and others. In this study, polymerase chain reaction, followed by fluorescent in situ hybridization, was used for the visualization of hybridization signals at the cellular level, allowing the characterization of cell populations in the tissue samples. Our study provided a baseline molecular cytogenetics profile for Filipino retinoblastoma patients and insights on the malignancy of retinoblastoma, contributing to information on future diagnosis, prognosis, and therapeutics.

METHODS

Sample Source

Retinoblastoma tissue blocks were acquired from a histopathologic confirmed and clinically diagnosed retinoblastoma patient admitted in one of the local hospitals

in Metro Manila, Philippines. The tissue block specimens were anonymized, paraffinized, and subjected to slide preparation. Any data pertaining to the patient's identity were kept confidential and anonymous. Informed consent is not required from samples with no identifiers following the National Ethical Guidelines for Health and Health Related Research by Philippine National Health Research System Guidelines.[23]

The samples were manually sectioned to approximately 5 μm using a microtome (SLEE Medical), and mounted on glass slides pre-soaked in 0.1% HCl solution (3 to 5 minutes) coated with albumin. These were then incubated in an oven for 5 hours, set at 80˚C.

Hematoxylin and eosin (H&E) staining

The procedure for H&E staining was based on the protocol of the National Institute for Open Schooling with minor modifications.[24] Deparaffinization was performed through two changes of xylene for 5 minutes for each change. Rehydration of tissues was done by washing in decreasing concentrations of ethanol for 2 minutes for each change and finally in distilled water for 15 seconds. Slides were stained by hematoxylin for 6 minutes and excess stains were removed by washing with running tap water for 5 minutes. Differentiation of tissue slides was done in 1% acid alcohol for 15 seconds and rinsing was done with running tap water for 1 minute. Subsequently, slides were subjected to the bluing solution for 50 seconds and washed in running tap water for 5 minutes. Counterstaining was done by dipping the slides in 95% ethanol for 10x and soaking them in eosin Y for about 1 minute. Prior to mounting and viewing, the slides were subjected to dehydration by washing in 3 changes of 95% ethanol for 2.5 minutes each washing, and clearing with two washes of xylene for 2.5 minutes for each change. The prepared slides were viewed using a transmission light microscope (EVOS® XL Core, Life Technologies). A clinical pathologist from The Lung Center of the Philippines identified areas of the retina with cancer cell morphology and areas that have not been invaded by retinoblastoma.

Total RNA extraction

Total RNA was extracted from the areas with cancer tissue using the Total RNA Prep Kit Version 2.0 (BioFACT™) following the manufacturer's protocol. The concentration of the collected RNA was quantified using a spectrofluorometer (Quantus™).

Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR)

Amplification and quantification of expressed genes of CD133, MUC1, and KRT19 in retinoblastoma tissue samples were done using reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) (Bio-Rad). The Oligo(dT)20 Primer and SuperScript™ III Reverse Transcriptase kit (Medical Test Systems Inc.) were utilized to reverse transcribe the 10 ng total RNA into cDNA for 30 mins at 50˚C. The generated cDNA was amplified using SsoAdvanced™ Universal SYBR® Green Supermix kit (Bio-Rad). The cancer biomarker gene primer sequences and the utilized thermal profile for amplification are shown in Table 1.

Primers specific to the three genes were utilized along with the glyceraldehyde-3 phosphate dehydrogenase (GAPDH) gene as the reference. The relative expression of these four genes was determined using the delta-delta cycle threshold (ΔΔCt) method.[25]

Fluorescence in situ hybridization (FISH)

Tissue preparation

The FISH protocol of Cocadiz et al. and Richardson and colleagues was adopted with minor modifications in the deparaffinization and pre-treatment steps.[26,27] The deparaffinization step consisted of 3 washes of xylene for 5 minutes each, followed by two washes of 95% ethanol for 5 minutes each. The samples were then left to air dry.

Pre-treatment was done by soaking the deparaffinized slides in citrate buffer (0.04 M sodium citrate, 0.06 M citric acid; pH 6.0) in an 80˚C water bath for 10 minutes. This was followed by soaking in distilled water for 3 minutes. Excess buffer was removed through blotting.

Antigen retrieval and dehydration

Enzyme digestion was done by dropping approximately 2.0 ml of proteinase K (1 mg/mL) onto the sample on the slide. The slides were then incubated at 37˚C for 15 minutes, with the continuous dropping of proteinase K to prevent under-

Table 1. Primers Used to Amplify the Expressed Cancer Biomarker Genes and the Corresponding Thermal Profile

Cancer Biomarker		Primer sequence	Thermal Profile
GAPDH	Forward Primer	5'- ACC CAC TCC TCC ACC TTT G - 3'	95°C for 5 minutes (initial denaturation); 94°C for 0.5 minute (denaturation), 57°C for 0.5 minute (annealing), and 72°C for 0.5 minute, 35 cycles; 72°C for 5 minutes (final extension).
	Reverse Primer	5'- CTC TTG TGC TCT TGC TGG G - 3'	
CD133	Forward Primer	5'- GAC CAA AGA GGC GTT GGA GA - 3'	
	Reverse Primer	5'- TGG ACC AGG CCA TCC AAA TC - 3'	
MUC1	Forward Primer	5'- CTT ACA GCT ACC ACA GCC CC - 3'	
	Reverse Primer	5'- AGC TGG GCA CTG AAC TTC TC- 3'	
KRT19	Forward Primer	5'- ACT ACA GCC ACT ACT ACA CGA C - 3'	
	Reverse Primer	5'- CAG AGC CTG TTC CGT CTC AAA C - 3'	

Table 2. Cancer Biomarkers Used and its Gene Specific Fluorescent Probe Sequences

Cancer Biomarker	GenBank gene ID and chromosome location	Fluorophores	Probe Sequences
CD133	PROM1 (ID: 8842; NC_000004)	5'-FAM	TCC TGT GCA TGG TTG GGT AT
MUC1	MUC1 (ID: 4582; NC_000001)	5'-TET	CTG AGG CTG GAA AAC CAC TC
KRT19	KRT19 (ID: 3880; NC_000017)	5'-CY3	GGG GTT TAG AAT CTG CCC TC

digestion. This was followed by digestion at room temperature for another 15 minutes, then by washing with distilled water for 3 minutes. After air drying the slides, sequential washing with increasing concentrations of ethanol (70%, 85%, 95%) was done for 1 minute in each wash then left to air dry.

Hybridization

Primer3Plus software was used in designing gene specific fluorescent probes based from the GenBank sequences of *CD133*, *MUC1*, and *KRT19* genes. Probe specificity was verified using the Basic Local Alignment Search Tool (BLAST) software. Three fluorescent probe labels were used in hybridization: FAM (carboxyfluorescein), TET (tetrachlorofluorescein), and CY3 (cyanine-3).

Fluorescent probes were diluted with hybridization buffer and 8 µL aliquots of each probe mixtures were added onto the sample slides under dim light. Slides were then covered with glass coverslips and sealed without air bubbles. Co-denaturation was done by heating the slides in a container soaked in the water bath at 72˚C for 6 minutes. The slides were hybridized overnight for 16-18 hours at 37˚C in a humidified chamber. The design of fluorescent probes is shown in Table 2.

Post Hybridization

Slides were soaked in post-hybridization wash solution containing 100 mL saline-sodium citrate (SCC), 300 µL Tween 80, and penicillin-streptomycin with gentle agitation until to remove the coverslips. Subsequently, the slides were then placed in another post-hybridization wash (100 mL SSC, 300 µL Tween 80) and heated in a water bath for 2 minutes at 72˚C. The slides were left to air dry in the dark before applying 20 µL of 4',6-diamidino-2-phenylindole (DAPI) for nuclear counterstaining. These were then covered with a glass coverslip, sealed, and stored at -20˚C for 20 minutes before viewing. The slides were then viewed using a fluorescence microscope (EVOS® FL Color, Life Technologies). The copy number for each gene probe was determined using the protocol of Schildhaus and colleagues with modifications.[28] Cells with distinct borders using the DAPI stain were counted for the number of fluorescent signals inside the cell. Clusters of signals were given a count of 5. Counts were made by three observers and then averaged.

Waste Disposal

Tissue slides were placed in a red, rigid, leakproof, and puncture-resistant biohazard sharps container. The container was autoclaved at 121˚C for 30 minutes for decontamination.

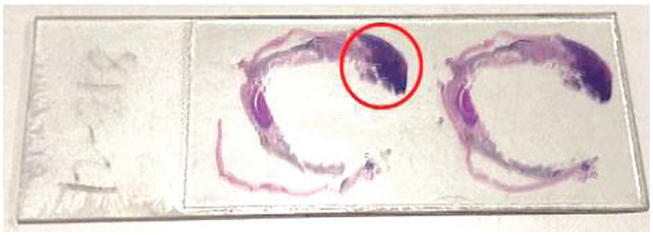

Figure 1. Fixed slide showing the hematoxylin and eosin staining of retinoblastoma samples. The encircled red area is the microsection of focus for examination where both neoplastic and non-neoplastic areas of the tissue can be seen.

RESULTS

Histopathological examination of tissues using H&E staining was applied to describe sections of tissues both the areas with cancer cells and the absence of neoplasm (Figure 1).

The tissue microsection of the patient's sample is presented in Figure 2. The retinoblastoma microsection revealed infiltration sheets, trabeculae, and nests of small blue cells with relatively uniform and hyperchromatic nuclei and scanty cytoplasm (Figure 2A). In some areas, Flexner-Wintersteiner rosettes were also seen, showing neoplastic cells lining up around an empty/central lumen delineated by distinct eosinophilic fibrous bars (Figure 2A; *white arrows with black outline*). Necrosis was observed in the adjacent areas. Horner-Wright rosette formation was also seen wherein a displaced cell nucleus is radially arranged about the tangle of fibrils. (Figure 2A; *black arrows with white outline*). In contrast, the non-neoplastic microsection revealed normal differentiation and distribution (Figure 2B) of pigmented epithelial cells and cell bodies of photoreceptors (Figure 2B; *gray arrows with white outline*). There is also the absence of Flexner-Wintersteiner rosette, a known neoplastic differentiation in the retinoblastoma sample.

Satisfactory probes signals were seen in both retinoblastoma and non-neoplastic microsections. The overlay of the signals detected in the retinoblastoma microsection using the three fluorescent probes (FAM, TET, CY3) is presented in Figure 3A. Nuclear DNA in both retinoblastoma (Figure 3B) and non-neoplastic section (Figure 3C) was labeled using DAPI. In the retinoblastoma microsection, multiple distinct signals can be observed for KRT19 (Figure 3D) and CD133 (Figure 3H) with only a few, distinct signals for MUC1 (Figure 3F). Conversely, only the CY3 probe has displayed

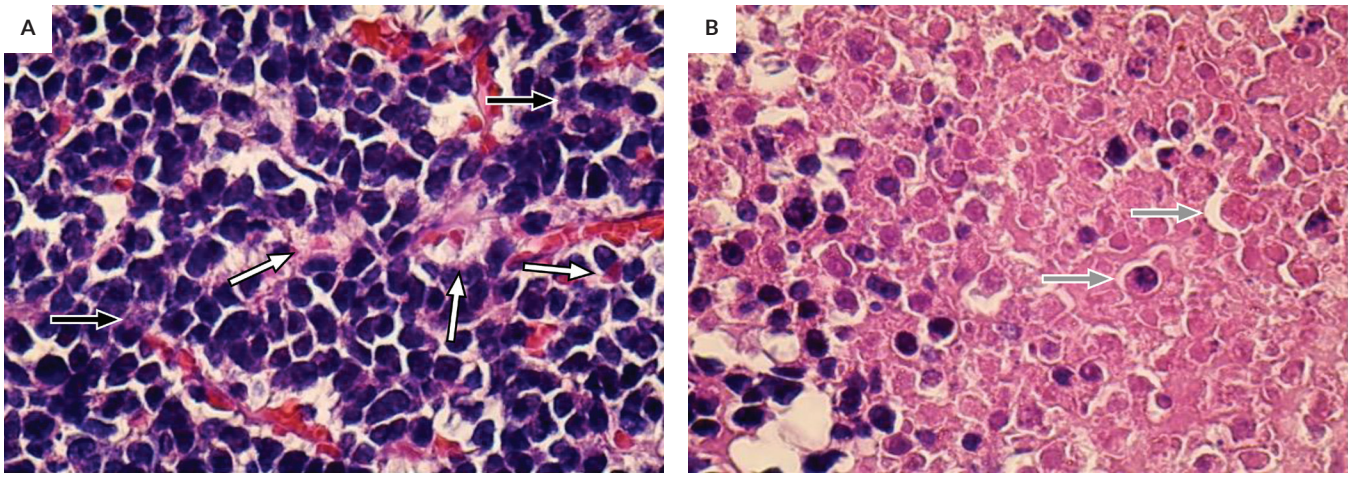

Figure 2. Tissue microsections in H&E staining viewed under transmission light microscopy (400x). **(A)** Retinoblastoma microsection; characterized by basophilic, poorly differentiated cells and in some areas, cuboidal cells forming a circle with a central lumen *(white arrows with black outline)* and with granular-stained center *(black arrows with white outline)* were also seen. **(B)** Non-neoplastic microsection; characterized by populations of pigmented epithelial cells and cell bodies of photoreceptors *(gray arrows with white outline)*.

Table 3. Summary of Copy Numbers of each Detected Gene by FISH, and Relative Gene Expression Determined by qPCR

Gene	Copy number (Average)		Relative Gene Expression of Rb
	Non-neoplastic	Retinoblastoma	
MUC1	0.45	2.91	~0.50
KRT19	1.93	1.90	~0.50
CD133	0.78	4.20	~1.50

multiple signals in the tested non-neoplastic tissue section indicating the presence of KRT19 in this section (Figure 3E). There were no TET (Figure 3G) and FAM (Figure 3I) probe signals found in the tested non-neoplastic tissue.

In terms of the average copy number of genes, *KRT19* is highly expressed in non-neoplastic tissue as compared to retinoblastoma. In contrast, the average copy number of *MUC1* and *CD133* were found higher in retinoblastoma in relation to the non-neoplastic tissue (Table 3). Furthermore, findings of the delta-delta cycle threshold (ΔΔCt) showed that *CD133* is approximately 1.5-fold overexpressed with respect to the non-neoplastic tissue (Figure 4). The relative gene expressions of both *MUC1* and *KRT19* are only slightly expressed in the retinoblastoma tissue with respect to the non-neoplastic tissue. The corresponding average copy number of genes and the relative gene expressions are summarized in Table 3.

DISCUSSION

Our results indicated that *CD133* was the most upregulated gene in the retinoblastoma sample as detected by increased copy number in FISH and supported by the relative gene expression analysis. This finding is most significant in considering the potential of retinoblastoma cells to undergo metastasis, as *CD133* has been found to be closely linked to the EMT mechanism of cancer cell migration.[12,13,29] As retinoblastoma is a rare disease commonly occurring in children, histopathological examination of the tumor remains to be the gold standard for definitive diagnosis. Likewise, the detection of *CD133* copy number via FISH especially in the early stages of retinoblastoma tumorigenesis will be useful in determining the progression of the disease and likewise will have an impact on the planning of therapeutic approaches.[30] However, there appears to be limited data on the utilization of FISH in the diagnosis and staging of retinoblastoma.

The use of FISH in the diagnosis of other cancers and tumors has been established.[31] FISH can also be used to determine predictive markers in non-small cell lung cancers through the identification of aberrant epidermal growth factor receptors and anaplastic lymphoma kinase genes.[32] Likewise, a case series reporting the diagnostic staging of spindle and round cell kidney tumors reported the utilization of FISH in differentially diagnosing rare renal tumors, when histopathological diagnosis remained unaffirmative.[33] The use of FISH in the diagnosis and assessment of the progression of retinoblastoma can then be considered.

Based on the reviewed literature, all three genes were found to be overexpressed in various cancers; the hypothesis is that it is the same in retinoblastoma cancers. Our results show that this is consistent for *CD133* but the copy number of *MUC1* and *KRT19* appeared to be similar between non-neoplastic and retinoblastoma cells. Among the three investigated genes, *CD133* appears to be the most viable marker as its copy number was supported by a higher relative gene expression data from qPCR.

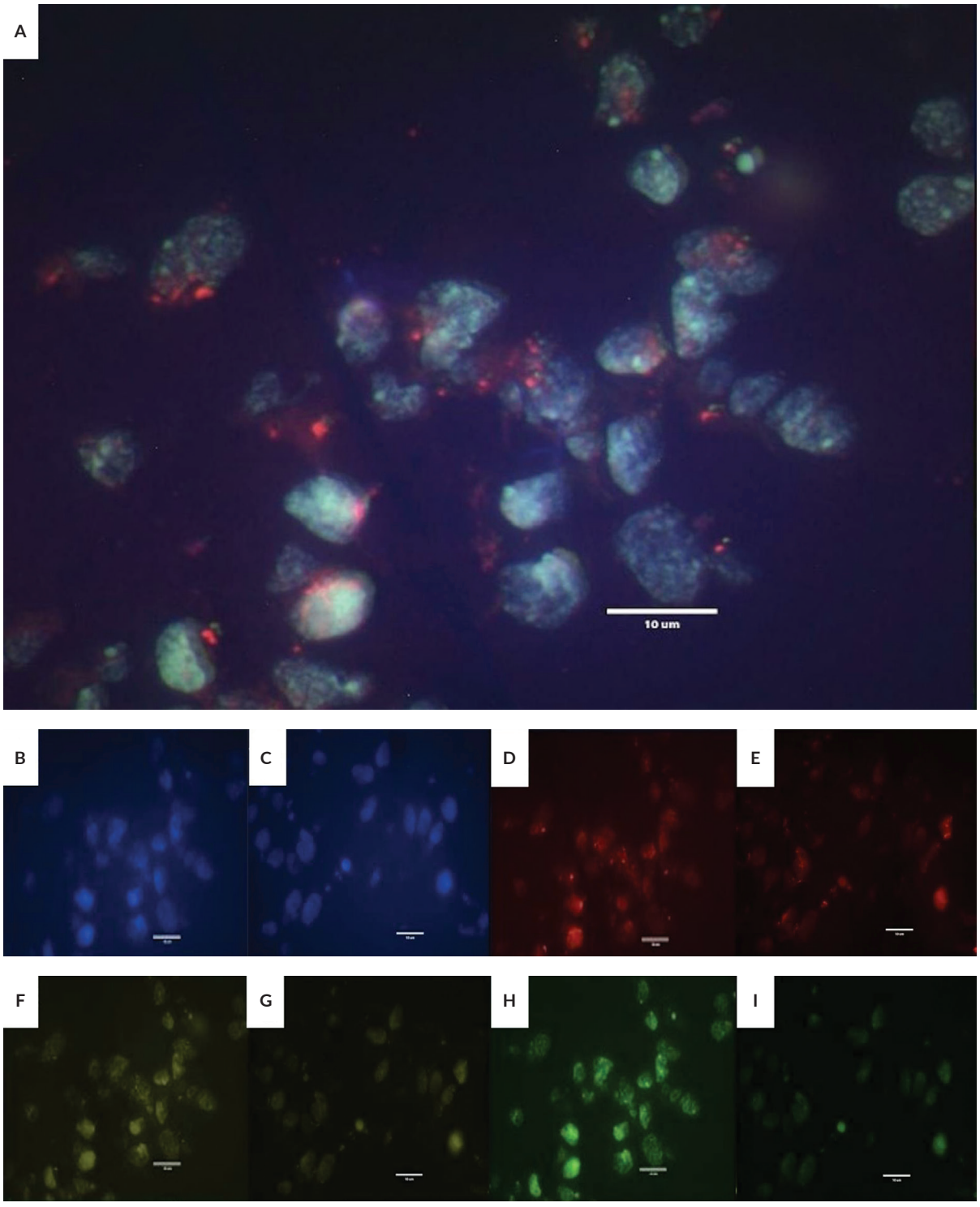

Figure 3. Fluorescence in situ hybridization using *FAM*, *CY3*, *TET* and *DAPI* (counterstain) probes of the retinoblastoma **(A-E)** and non-neoplastic microsections **(F-I)** under fluorescence microscope (400x). **(A)** Overlay signals of *FAM*, *CY3*, *TET*, and *DAPI* in retinoblastoma microsection. *DAPI* probe used in the localization of nucleus as seen in bright blue spots against the field: **(B)** retinoblastoma microsection; **(C)** non-neoplasm microsection. *CY3* stain for detection of *KRT19*, signals are seen as bright red dots: **(D)** retinoblastoma microsection; **(E)** non-neoplasm microsection. *TET* stain for detection of *MUC1*, signals are seen as bright yellow dots: **(F)** retinoblastoma microsection; **(G)** non-neoplasm microsection. *FAM* stain for detection of *CD133*, signals are seen as bright green dots: **(H)** retinoblastoma microsection; **(I)** non-neoplasm microsection. Scale, 10 µm; magnification, 400x.

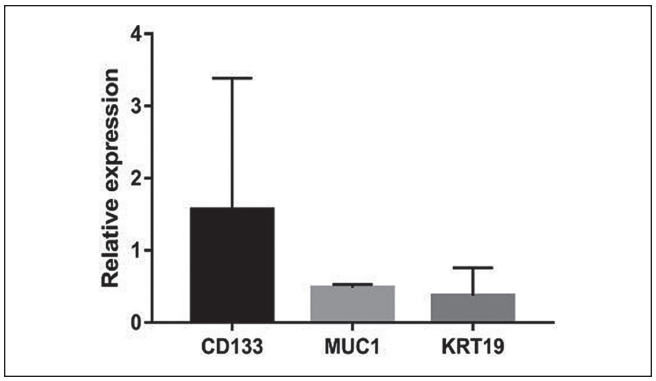

Figure 4. Gene expression of *CD133*, *MUC1*, and *KRT19* in retinoblastoma normalized against *GAPDH*. Relative gene expression values were calculated using delta Ct. (2^n).

Using FISH in cancer diagnostics can be beneficial when assessing tumors with low cell numbers.[34] As such, retinoblastoma tumors may yield small sample sizes due to the rarity of the disease and can thus be used in FISH to determine gene copy duplication for assessment. Aside from this, FISH as a method in determining the severity of cancer can be used to plan treatment as prompt intervention is necessary in order to save the lives of patients.[4] Since tumor biopsies of retinoblastoma are generally contraindicated, the assessment of aqueous humor as a surrogate for a tumor biopsy can be done. Analysis of aspirated aqueous humor in retinoblastoma patients has shown a significant amount of isolated nucleic material,[35] opening the possibility of taking cells isolated from the aqueous humor and subjecting it to FISH analysis. However, previous studies recommend targeted evaluation of cells in order to take full advantage of FISH,[31] that is, the prerequisite knowledge is necessary for assessing cancer cells as opposed to normal cells or non-malignant cells.

Prognostication is another important component in the clinical management of cancer patients. Approaches to prognosis are therefore continually being explored and developed for different types of cancer. One strategy for both diagnosis and prognosis of cancer makes use of the detection of genes as molecular biomarkers. Because of this versatility and the growing evidence for its value in cancer diagnostics and prognostics, many studies have looked into the use of FISH for genetic aberration detection in specific types of tumors. For example, a study compared the value of conventional cytology and FISH-detected chromosomal aberration in predicting failure to intravesical bacillus Calmette-Guerin (BCG) therapy for non-muscle-invasive bladder cancer and concluded that FISH was superior to cytology both as a marker of relapse and in the assessment of the failure risk after BCG.[36] In a prospective study, a strong association was noted between the prevalence of human chromosome telomerase gene expression and cervical intraepithelial neoplasia 1 (CIN 1) natural prognosis using FISH as their gene detection method.[37] FISH analysis was also performed for the mixed-lineage leukemia gene using marrow samples from patients with acute leukemia. This demonstrated that FISH could detect aberrant MLL signals which were associated with prognosis for patients with acute myeloid leukemia.[38]

Although FISH continues to prove itself as a promising prognostication method for cancers, it has its share of limitations. For instance, FISH detects only abnormalities in genomic regions targeted by the specific probes and does not use a whole-genome approach unlike in a full metaphase karyotype via G-bands.[38,39] In this study, only *CD133*, *KRT19*, and *MUC1* were explored as potential markers and only *CD133* had a positive result. The utility of FISH may therefore be recommended for confirmatory instead of exploratory studies for genetic markers for retinoblastoma. FISH is also unable to detect missense/nonsense point mutations, very small insertions, and deletions, as well as very small intragenic duplications.[40] Alcohol-fixed or air-dried specimens are more well-suited for FISH rather than formalin-fixed and paraffin-embedded cell block or tissue sections, since cells from the former showed less nuclear overlap, lack nuclear truncation, and generally have better DNA quality.[35] Paraffin-embedded cell blocks were used in this study and the appearance of cloudy clusters may have been influenced by possible nuclear overlaps and poor DNA quality. It is therefore recommended that optimization for each type of specimen be done for FISH to be well-suited for all types and data is not skewed by the differences in specimen preservation methods. Additionally, it is also noted that although cytology is ideally suited for FISH applications, it is crucial to involve cytopathologists and cytotechnicians to have the best positive results.[31] Further studies need to be done to explore the use of FISH for determining the prognosis of retinoblastoma.

Nonetheless, FISH using genetic material remains to be a good supplementary or even alternative approach in prognostication. It allows for higher resolution analysis of structural chromosomal rearrangements specific to acute myeloid leukemia which cannot be detected using G-banded karyotyping, the gold standard method for diagnosis and prognosis of the said disease.[40] FISH has been used as an ancillary approach for prognosis investigation in esophageal adenocarcinoma. A study developed a multi-color FISH assay that is easy to use and ready for clinical application on formalin-fixed, paraffin-embedded material. It combines the sensitivity, specificity, and diagnostic value of *CDKN2A*, *ZNF217*, *ERBB2*, and *MYC* and the prognostic value of *ZNF217* and *ERBB2* which is claimed to be superior to single markers.[41] Currently, FISH-cytogenetics is utilized in the development of a biomarkers-only prognostic model for patients with chronic lymphocytic leukemia (CLL).[42]

CONCLUSION AND RECOMMENDATIONS

The results of the study suggest that *CD133* can be potentially used as a retinoblastoma marker due to its increased

copy number and gene expression in the tissues. Although *MUC1* and *KRT19* have been observed to be overly expressed in other cancers, their low copy number and gene expression in retinoblastoma propose that they may not be ideal markers for this cancer. Overall, the study lends support to the possible use of fluorescent in situ hybridization of *CD133* as a diagnostic tool and assessment of its potential for metastasis in retinoblastoma. Further studies on the optimization of fluorescent in situ hybridization can be performed. We recommend that the *CD133* copy number and expression be compared across the different retinoblastoma stages and a retinoblastoma cell line (e.g. RB116). Other genetic markers such as centromere protein E, lamin B1 protein, cell division cycle protein 20, etc. for retinoblastoma may also be explored.

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All authors certified fulfillment of ICMJE authorship criteria.

Author Disclosure

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REFERENCES

1. Shields CL, Fulco EM, Arias JD, Alarcon C, Pellegrini M, Rishi P, et al. Retinoblastoma frontiers with intravenous, intra-arterial, periocular, and intravitreal chemotherapy. Eye (Lond). 2013 Feb;27(2): 253-64. doi: 10.1038/eye.2012.175.
2. Siegel RL, Miller KD, Fuchs HE, Jemal A. Cancer statistics, 2022. CA Cancer J Clin. 2022 Jan;72(1):7-33. doi: 10.3322/caac.21708.
3. Guevarra MCB, Eala MAB, Dee EC, Mercado GJV, Collantes ERA. Looking through the scope: retinoblastoma in the Philippines. Eye (Lond). 2022 Dec;36(12):2356-7. doi: 10.1038/s41433-022-02069-4.
4. Kumar V, Abbas AK, Fausto N, Aster JC. Robbins and Cotran Pathologic Basis of Disease. 9th ed. Canada: Elsevier health sciences; 2015. p. 1339.
5. Dimaras H, Corson TW, Cobrinik D, White A, Zhao J, Munier FL, et al. Retinoblastoma. Nat Rev Dis Primers. 2015 Aug;1:15021. doi: 10.1038/nrdp.2015.21.
6. Mendoza PMR, Tuano PMC, Verzosa LG, Huerto RFS, Enriquez MLD, Macias EL. Molecular genetic profiling of Filipino patients with retinoblastoma: a preliminary study. Philipp J Ophthalmol. 2012 Jan-Jun;37:39-44.
7. Kudinov AE, Karanicolas J, Golemis EA, Boumber Y. Musashi RNA-binding proteins as cancer drivers and novel therapeutic targets. Clin Cancer Res. 2017 May;23(9):2143-53. doi: 10.1158/1078-0432.CCR-16-2728.
8. Zhao J. Cancer stem cells and chemoresistance: The smartest survives the raid. Pharmacol Ther. 2016 Apr;160:145-58. doi: 10.1016/j.pharmthera.2016.02.008.
9. Tang Z, Ma H, Mao Y, Ai S, Zhang P, Nie C, et al. Identification of stemness in primary retinoblastoma cells by analysis of stem-cell phenotypes and tumorigenicity with culture and xenograft models. Exp Cell Res. 2019 Jun;379(1):110-118. doi: 10.1016/j.yexcr.2019.03.034.
10. Zhou HM, Zhang JG, Zhang X, Li Q. Targeting cancer stem cells for reversing therapy resistance: mechanism, signaling, and prospective agents. Signal Transduct Target Ther. 2021 Feb 15;6(1):62. doi: 10.1038/s41392-020-00430-1.
11. Nair RM, Balla MM, Khan I, Kalathur RKR, Kondaiah P, Vemuganti GK. In vitro characterization of CD133lo cancer stem cells in Retinoblastoma Y79 cell line. BMC Cancer. 2017 Nov 21;17(1): 779. doi: 10.1186/s12885-017-3750-2.
12. Nomura A, Banerjee S, Chugh R, Dudeja V, Yamamoto M, Vickers SM, et al. CD133 initiates tumors, induces epithelial-mesenchymal transition and increases metastasis in pancreatic cancer. Oncotarget. 2015 Apr;6(10):8313-22. doi: 10.18632/oncotarget.3228.
13. Maeda K, Ding Q, Yoshimitsu M, Kuwahata T, Miyazaki Y, Tsukasa K, et al. CD133 modulate HIF-1α expression under hypoxia in EMT phenotype pancreatic cancer stem-like cells. Int J Mol Sci. 2016 Jun;17(7):1025. doi: 10.3390/ijms17071025.
14. Nath S, Mukherjee P. MUC1: a multifaceted oncoprotein with a key role in cancer progression. Trends Mol Med. 2014 Jun;20(6): 332-42. doi: 10.1016/j.molmed.2014.02.007.
15. Gao T, Cen Q, Lei H. A review on development of MUC1-based cancer vaccine. Biomed Pharmacother. 2020 Dec;132:110888. doi: 10.1016/j.biopha.2020.110888.
16. Jo H, Brito S, Kwak BM, Park S, Lee MG, Bin BH. Applications of mesenchymal stem cells in skin regeneration and rejuvenation. Int J Mol Sci. 2021 Feb 27;22(5):2410. doi: 10.3390/ijms22052410.
17. Wang XM, Zhang Z, Pan LH, Cao XC, Xiao C. KRT19 and CEACAM5 mRNA-marked circulated tumor cells indicate unfavorable prognosis of breast cancer patients. Breast Cancer Res Treat. 2019 Apr;174(2):375-85. doi: 10.1007/s10549-018-05069-9.
18. Giovanella L, Imperiali M, Trimboli P. Role of serum cytokeratin 19 fragment (Cyfra 21.1) as a prognostic biomarker in patients with differentiated thyroid cancer. Sci Rep. 2017 Aug;7(1):7359. doi: 10.1038/s41598-017-07915-0.
19. Zielińska KA, Katanaev VL. The signaling duo CXCL12 and CXCR4: chemokine fuel for breast cancer tumorigenesis. Cancers (Basel). 2020 Oct;12(10):3071. doi: 10.3390/cancers12103071.
20. Subramaniyan V, Fuloria S, Gupta G, Kumar DH, Sekar M, Sathasivam KV, et al. A review on epidermal growth factor receptor's role in breast and non-small cell lung cancer. Chem Biol Interact. 2022 Jan 5;351:109735. doi: 10.1016/j.cbi.2021.109735.
21. Yao H, Yang Z, Liu Z, Miao X, Yang L, Li D, et al. Glypican-3 and KRT19 are markers associating with metastasis and poor prognosis of pancreatic ductal adenocarcinoma. Cancer Biomark. 2016;17(4): 397-404. doi: 10.3233/CBM-160655.
22. Merlos-Suárez A, Barriga FM, Jung P, Iglesias M, Céspedes MV, Rossell D, et al. The intestinal stem cell signature identifies colorectal cancer stem cells and predicts disease relapse. Cell Stem Cell. 2011 May 6;8(5):511-24. doi: 10.1016/j.stem.2011.02.020.
23. Philippine Health Research Ethics Board. National ethical guidelines for health and health-related research [Internet]. 4th ed. 2017 [cited 2023 Mar]. 55 p. Available from: https://ethics.healthresearch.ph/index.php/phoca-downloads/category/4-neg
24. National Institute of Open Schooling. Hematoxylin and Eosin Staining [Internet]. 2008 [cited 2023 Mar]. 62 p. Available from: https://nios.ac.in/media/documents/dmlt/HC/Lesson-10.pdf
25. Zhang JD, Ruschhaupt M, Biczok R. ddCt method for qRT–PCR data analysis. [Internet]. 2013 Apr 4 [cited 2023 Mar]. Available from: http://bioconductor.statistik.tu-dortmund.de/packages/3.6/bioc/vignettes/ddCt/inst/doc/rtPCR.pdf.

26. Cocadiz JAM, Lim CCY, Dalet JT, Barzaga MTA, Heralde III FM. CD133, MUC1, and KRT19 chromosomal rearrangements and gene expressions as potential biomarkers for liver cancer. Int J Med Res Health Sci. 2017;6(9):21-31.
27. Richardson SO, Huibers MMH, de Weger RA, de Leng WWJ, Hinrichs JWJ, Meijers RWJ, et al. One-fits-all pretreatment protocol facilitating Fluorescence In Situ Hybridization on formalin-fixed paraffin-embedded, fresh frozen and cytological slides. Mol Cytogenet. 2019 Jun;12:27. doi: 10.1186/s13039-019-0442-4.
28. Chazotte B. Labeling nuclear DNA using DAPI. Cold Spring Harb Protoc. 2011 Jan;2011(1):pdb.prot5556. doi: 10.1101/pdb.prot5556.
29. Schildhaus HU, Heukamp LC, Merkelbach-Bruse S, Riesner K, Schmitz K, Binot E, et al. Definition of a fluorescence in-situ hybridization score identifies high- and low-level FGFR1 amplification types in squamous cell lung cancer. Mod Pathol. 2012 Nov;25(11):1473-80. doi: 10.1038/modpathol.2012.102.
30. Tu Z, Xie S, Xiong M, Liu Y, Yang X, Tembo KM, et al. CXCR4 is involved in CD133-induced EMT in non-small cell lung cancer. Int J Oncol. 2017 Feb;50(2):505-14. doi: 10.3892/ijo.2016.3812.
31. Sugita S, Hasegawa T. Practical use and utility of fluorescence in situ hybridization in the pathological diagnosis of soft tissue and bone tumors. J Orthop Sci. 2017 Jul;22(4):601-12. doi: 10.1016/j.jos.2017.02.004.
32. Abbas M, Dämmrich ME, Braubach P, Kramer MW, Grünwald V, Merseburger AS, et al. Role of immunohistochemistry and fluorescence in-situ hybridization (FISH) in the diagnosis of spindle and round cell tumors of the kidney. J Egypt Natl Canc Inst. 2015 Sep;27(3):173-8. doi: 10.1016/j.jnci.2015.04.005.
33. Yan X, Ma L, Yi D, Yoon JG, Diercks A, Foltz G, et al. A CD133-related gene expression signature identifies an aggressive glioblastoma subtype with excessive mutations. Proc Natl Acad Sci U S A. 2011 Jan;108(4):1591-6. doi: 10.1073/pnas.1018696108.
34. Tamura K, Aoyagi M, Ando N, Ogishima T, Wakimoto H, Yamamoto M, et al. Expansion of CD133-positive glioma cells in recurrent de novo glioblastomas after radiotherapy and chemotherapy. J Neurosurg. 2013 Nov;119(5):1145-55. doi: 10.3171/2013.7.JNS122417.
35. Berry JL, Xu L, Murphree AL, Krishnan S, Stachelek K, Zolfaghari E, et al. Potential of aqueous humor as a surrogate tumor biopsy for retinoblastoma. JAMA Ophthalmol. 2017 Nov;135(11):1221-30. doi: 10.1001/jamaophthalmol.2017.4097.
36. Ng K, Stenzl A, Sharma A, Vasdev N. Urinary biomarkers in bladder cancer: A review of the current landscape and future directions. Urol Oncol. 2021 Jan;39(1):41-51. doi: 10.1016/j.urolonc.2020.08.016.
37. Li L, Jiang W, Zeng SY, Li L. Prospective study of hTERC gene detection by fluorescence in situ hybridization (FISH) in cervical intraepithelial neoplasia 1 natural prognosis. Eur J Gynaecol Oncol. 2014;35(3):289-91.
38. Gersen SL. The principles of clinical cytogenetics [Internet]. 3rd ed. New York: Springer Science+ Business Media New York; 2013 [cited 2023 Mar 29]. 326 p. Available from: https://link.springer.com/book/10.1007/978-1-4419-1688-4
39. Zneimer SM. Cytogenetic Abnormalities: Chromosomal, FISH, and Microarray-Based Clinical Reporting and Interpretation of Result. United Kingdom: John Wiley & Sons; 2014 Sep 22 [cited 2023 Mar 29]. 231 p. Available from: https://onlinelibrary.wiley.com/doi/epdf/10.1002/9781118412602.ch15
40. Gonzales PR, Mikhail FM. Diagnostic and prognostic utility of Fluorescence In situ Hybridization (FISH) analysis in acute myeloid leukemia. Curr Hematol Malig Rep. 2017 Dec;12(6):568-573. doi: 10.1007/s11899-017-0426-6.
41. Geppert CI, Rümmele P, Sarbia M, Langer R, Feith M, Morrison L, et al. Multi-colour FISH in oesophageal adenocarcinoma-predictors of prognosis independent of stage and grade. Br J Cancer. 2014 Jun;110(12):2985-95. doi: 10.1038/bjc.2014.238.
42. Delgado J, Doubek M, Baumann T, Kotaskova J, Molica S, Mozas P, et al. Chronic lymphocytic leukemia: A prognostic model comprising only two biomarkers (IGHV mutational status and FISH cytogenetics) separates patients with different outcome and simplifies the CLL-IPI. Am J Hematol. 2017 Apr;92(4):375-80. doi: 10.1002/ajh.24660