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STUDY TITLE:

11372 - ~~Molecular profiling of Sarcoma – a comprehensive analysis on five cases of Ewing’s sarcoma from a tertiary cancer care centre~~

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Molecular profiling of Sarcoma - a comprehensive analysis on five cases of Ewing’s sarcoma from a tertiary cancer care centre
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Abstract:

Background - Ewing Sarcoma (ES) is a biologically aggressive family of tumors of bone and soft tissue, affecting adolescents and young adults, between the age group of 5-25, and is the second most common cancer in children. In early stage diagnosis, the 5-year survival rate is of 73%; however, in a metastatic condition the survival rate is only 20-30%, with an early recurrence within first 2 years. Beyond the classic translocation EWS-FLI1, there are no validated biomarkers or drug targets in ES. Although, there have been continuous improvements in the first line therapy based on the experience from various clinical trials, options for second line therapy are limited. This pilot study aims

to derive new oncogenic drivers in recurrent ES which could be potential drug targets after clinical validation.

Methods - Five known cases of ES diagnosed with histopathology and immunohistochemistry were chosen for this study. DNA was isolated from tumors using QIAamp DNA Mini Kit, for performing the PCR sequencing. Targeted Re-sequencing of tumor DNA was performed using Ion Ampliseq™ Cancer Panel V 1.0 on Ion Torrent Next-Generation Sequencing platform (NGS) (Life Technologies, Carlsbad, CA). Torrent Suite Software (TSS), version 2.0 was used for all the sequence analysis.

Results - We identified several common germ line/somatic driver/predisposing mutations in all 5 cases of ES, suggesting a common underlying mechanism. A homozygous, germ line polymorphism in APC gene (COSM19349) which was earlier identified in FAP was a unique observation in all the Ewing's' tumors, suggesting a possible role of genetic predisposition. Another common characteristic feature was the somatic mutation C382R (COSM36906) in FGFR2 gene in its transmembrane anchor domain, and is the first ever observation in ES. As transmembrane anchor plays an important role in FGFR2 activation, this mutation may lead to damaging effect on the function of the protein. Another important finding was the presence of Q61R (COSM583; COSM584; COSM582; COSM12725; COSM579) mutation in NRAS, E545D (COSM27374; COSM765) in PIK3CA in all the cases, suggesting alterations in downstream EGFR pathway and hence a possible role for mTOR inhibitors.

Conclusions -

Taken together, this pilot study on Ewing's' sarcomas has identified possible role of APC, FGFR2 and EGFR pathway (NRAS/PIK3CA) as potential oncogenic drivers/predisposition markers in this class of tumors. We have identified a footprint of 14 common mutations in all the five cases of Ewings Sarcoma, which has potential application in personalized management of this disease. However, further clinical investigation and extensive validation is warranted for these biomarkers to be approved as successful drug targets.

Introduction:

~~Ewing sarcoma (ES) belongs to the group of bone and soft tissue neoplasm's commonly referred to as small, round, blue-cell tumors of childhood. Nearly, 20-30% of patients present with metastatic disease (1-3). Radical resection followed by multidrug chemotherapy that includes vincristine, doxorubicin, ifosfamide and etoposide are the standard treatment of choice with/without radiation (4-8). In spite of the available treatment options, 80% of the Ewing's Sarcoma recurs within 2 years of diagnosis and the prognosis is poor with a 5 year survival of less than 20% (1-3). Although, some case reports have demonstrated that myeloablative therapy is better than standard chemotherapy followed by allogeneic stem cell transplantation, nevertheless in large scale studies it has not shown improvement in survival outcome (9-12). Presence of clinically non detectable metastases/micro metastases and the aggressive biological behavior of the tumor are some of the factors thought to contribute to early recurrence. There is no~~

standardized second line treatment for relapsed or refractory ES. Hopefully, further molecular characterization of the tumors may add value to the response rate by selecting patients who would likely benefit from options such as targeted therapy as part of the combinatorial regimen, thus improving clinical outcomes.

Current Treatment Modalities

Chemotherapy/Surgery/Radiation:

It is well established that chemotherapy is the mainstay of treatment in Ewing's sarcoma and is a necessary addition to local control in order to achieve a reasonable expectation of cure. The treatment plan generally consists of three stages: initial cytoreduction with chemotherapy to eradicate micro metastatic disease and facilitate effective local control measures with wide negative margins; definitive radiation or surgical therapy to eradicate all known disease; and consolidation therapy for eradication of occult residual disease to reduce the likelihood of tumor recurrence. Importantly, neoadjuvant chemotherapy not only helps to achieve optimal cytoreduction to facilitate limb salvage procedures but also provides a chance to assess the response to chemotherapy (13, 14).

Multidrug chemotherapy for Ewing sarcoma mostly always includes vincristine, doxorubicin, ifosfamide, cyclophosphamide and etoposide (4-8, 15). Certain protocols incorporate dactinomycin. The mode of administration and dose intensity of cyclophosphamide within courses differs markedly between protocols. An European Intergroup Cooperative Ewing Sarcoma Study (EICESS) trial suggested that 2 grams of cyclophosphamide produced a similar event free survival (EFS) compared to 6 grams of ifosfamide in patients with lower risk disease, and thus, identified a trend towards better DFS for patients with localized Ewing sarcoma and higher risk disease when treatment included etoposide (GER-GPOH-EICESS-92). Protocols in the United States generally alternate courses of vincristine, cyclophosphamide, and doxorubicin with courses of ifosfamide /etoposide, while European protocols generally combine vincristine, doxorubicin, and an alkylating agent with or without etoposide in a single treatment cycle (15).

Existing Biomarker based therapy:

The underlying genetic modifications that effect oncogenesis are most often reflected with unique histological and molecular sub-categories. Therefore, understanding the diversity in the molecular subtypes particularly with the variety and frequency of mutations could answer some of the potential challenges in addressing novel biomarker driven combinatorial anticancer therapy. Hence, the need for high throughput multi gene profiling to understand the

structure and function of genes associated with various cancers, thus, providing the guiding rules for clinical management in the era of personalized medicine.

Patient selection using biomarkers and understanding response and resistance mechanisms in relation to IGF1R and mammalian target of rapamycin pathways are areas of active research. Specifically, inhibition of insulin like growth factor 1 receptor (IGF1R) signaling and the mammalian target of rapamycin (mTOR) pathways have emerged as a targeted therapy in ES, with selected patients experiencing dramatic therapeutic responses. However, targeted therapies in general, and these responders in particular, are faced with the ultimate conundrum of eventual resistance. To optimize the response, combination of IGF1R and mTOR inhibitor-based regimens along with chemotherapy, in the upfront setting in newly diagnosed high-risk ES could predict the true benefit of IGF1R inhibitors in these patients (16-20). In addition, angiogenic markers have also been considered in the combined regimen (21-22). Unfortunately, the clinical response data are relatively scarce and hence difficult to interpret.

Molecular classification in ESFT: Until date, beyond histology, the diagnosis of Ewing's Sarcoma is supported by the immunohistochemical analysis for CD99 expression in correlation with other clinical and pathologic parameters. However, the expression of CD99 is not limited to Ewing's Sarcoma, as it is increasingly expressed in other malignancies such as synovial sarcoma, non Hodgkin lymphoma and GIST, thus making it a relatively non-specific marker. The cell of origin based on histology and IHC is very poorly understood in ES as compared to other sarcomas like osteosarcoma and liposarcoma which show lineage-specific differentiation (23-25). Several studies support two different subtypes/lineage of origin: the mesenchymal as well as neuro-ectodermal origin, and both the subtypes have a common underlying chromosomal re-arrangement, which results in a group of translocations in this class of tumors driven by EWS1 gene, a TET family transcription regulator. The chimeric partner involved in this translocation is most often a DNA binding protein, which belongs to ETS family of transcription factors. The translocation t(11, 22) present in 85% of the ES tumors, results in an oncogenic fusion protein with N-terminus of EWS and C-terminus of FLI1 gene, that works as a transcriptional regulator (23-25). Whether it is an initial event or an early mediator for malignant transformation is not clearly understood and the complexity associated with the functional role of EWS-FLI1 protein, makes it a major therapeutic challenge (26). Taken together, the diagnostic challenge with a single IHC marker and redundancy in histo-molecular classification available so far based on the EWS1 interacting partners and poor

understanding on the biology of recurrence, has limited the improvement in clinical management of this class of tumors.

Our approach:

It has been well established that, cancer is caused by an accumulation of mutations in genome. Some of the recent reports addressing the genomic/proteomic landscape of Primary Ewing sarcoma and its biology have revealed the presence of novel mutations in STAG2 and TP53 genes and translocations involving FOXO4 and NCAT (27-30). Considering the heterogeneity in the gene pool and various other etiological factors addressing the tumor onset and behavior, further studies are warranted to understand unique pathways driving oncogenesis in this class of tumors.

Many genes, particularly kinases and transcriptional regulators/tumor suppressors have been associated with tumor progression either through germ line or somatic mutations. Kinase inhibition is one popular therapeutic modality for treating human malignancy (31). However, little is known about the kinases critical to the development and its therapeutic role in many pediatric solid tumors such as Ewing sarcoma (32). In order to derive new therapeutic options and further molecular classification of recurrent ES, this pilot study is focused on screening a panel of mutations in 46 cancer related genes, primarily involving ser/thr/tyr kinases and transcription factors/tumor suppressors using Next Generation Sequencing technologies, thus providing insight on the different mechanisms in Ewing's sarcomas

Methods and Patient sample selection

Tumor DNA from all the five cases of ES was assessed for tumor load. FFPE tumor tissue blocks which had more than 40% tumor burden were selected for DNA isolation. The tumor genomic DNA was isolated from 2x20micron section using QIA Amp DNA Mini kit as per the manufacturer's recommendation. The DNA was assessed for quality and quantity using Nano drop as well as fluorescence dye binding assay using Cubit platform.

Selection of the 46 gene Ampliseq panel and the Assay Design

46 gene Ampliseq cancer panel (V1.0) was chosen for the screening of mutations in this study. This panel was designed based on the mutational spectrum in various cancers as documented in multiple databases such as the COSMIC database from Sanger Institute, The Cancer Genome Atlas and from various scientific reports indexed in the Pub Med literature. We queried for known somatic mutations in proto-oncogenes and oncogenes which are primarily tumor suppressors, transcriptional regulators and kinases. The panel consists of frequently occurring mutations such as SNPs, Insertions and deletions across cancer subtypes, including the genes related to therapeutic benefit that has been clinically validated in other malignancies.

Genotyping on the Ion-torrent platform PGM

Validated Primers pool set of the Ampliseq Cancer Panel was procured from Invitrogen Bioservices pvt ltd. These primers were earlier validated on human cancer lines with known mutational status as well as on the control DNA derived from the human Hap map DNAs (Coriell institute) [important reference for the ampliseq panel]. Genomic DNA was quantified using Qubit platform with dsDNA dye binding Assay Kit (Invitrogen, Carlsbad, California), subjected to bar-coding and library preparation using specific kits. Minimum of 10ng of DNA as quantified by Qubit was taken as input for generation of genomic libraries. The tumor samples were barcode multiplexed using specific primers from life technologies. The bar-coded PCR libraries were then purified and then subjected to target enrichment using emulsion PCR technologies. The Emulsion PCR product was further purified to clear any non amplified libraries and other PCR impurities and then loaded on the Ion 316 Semiconductor chip for sequencing. The Sequencing run was performed after a quality check on the four nucleotide run TCAG for an average of 50 bases, beyond which the run continued. The raw data output from the PGM sequencer was further analyzed used the Torrent Suit Software version 2.0.

(The genotyping of the PCR products was performed as previously described [functional of PGM sequencer, reference].)

Assembly and analysis of the raw data using torrent suite software: The raw data output from the Ion-torrent instrument was passed through several iterative cycles of sequence alignment based on the raw data profiles with quality control filters and those regions where the Q20 value was satisfactory was considered for further alignment and analysis (33-34). The variants were analyzed by variant caller software and the functional role of variants was predicted using SIFT/PROVEAN, Mutation assessor, Polyphen-2 suite of programs (35-39)

Ethics Statement

Institutional review board approval was obtained to retrospectively study these tumor samples from the Central Ethics Committee, HCG Cancer Hospitals, Bangalore. The protocol number is XXXXX. The institutional review board waived the need to individual patient consent as the samples were all de-identified. Tumor specimens stored in the form of formalin-fixed paraffin embedded blocks were obtained from the clinical archives of the Hospital Tissue Repository

Results

Characteristics of Clinical Tumor Samples

Five recurrent cases of soft tissue tumors, wherein, the diagnosis was highly suggestive of Ewing's sarcoma, based on histology and immunohistochemistry were selected for this study.

NGS analysis

Targeted Re-sequencing of tumours was performed using Ion Ampliseq™ Cancer Panel V1.0 on Ion Torrent Next-Generation Sequencing platform (NGS) (Life Technologies, Carlsbad, CA). The panel is designed to scan hotspot regions on 46 cancer related genes in 190 amplicons, screening for 739 mutations. The data throughput provided a median depth of coverage of 1200X with Q20 accuracy, facilitating detection of rare somatic mutations, insertions and deletions in patient samples. Torrent Suite Software (TSS), version 2.0 was used for all the sequence analysis. The panel comprised of oncogenes and tumour suppressor genes that have been previously implicated in the tumorigenesis in other malignancies, in order that the findings to could be well adopted in ES after successful clinical validation.

Mutation Analysis

The summary of mutations observed in both kinases and tumor suppressor oncogenes, wherein a subset of them has been predicted to be likely pathogenic is described in Table 1. We specifically included an experimental criterion while performing NGS analysis, which is programmed for deep sequencing, implying every base is covered with > 1000X. All the samples had at least one oncogenic driver mutation identified. The presence of unique homozygous germ line polymorphism (rs41115) in the APC gene suggests its possible role as a predisposing event for Ewing's sarcoma. On the similar lines, single nucleotide insertions in APC and TP53 resulting in altered function of these proteins may also suggest a combined role in initiation of tumorigenesis.

A total of 79 mutations were detected among which synonymous were 36 and non-synonymous were 43. Number of alternations in coding variants was 40 and 2 in the intronic region and 1 in the 3' UTR. There were 27 non-synonymous variants which were predicted to be neutral/low in significance in perturbing the normal functional role of these genes.

Single nucleotide Insertions in APC (T1493fs*21) and TP53 (T254fs*10) which results in frame shift elongation of the protein molecules was commonly observed in all the Ewing's cases, and are the novel findings in this study. Beyond the mutations described in tumor suppressors, there were other mutations identified as part of the cancer gene panel. These genetic alterations include: NRAS (Q61R/P/K), PIK3CA (E545D, E707K), FGFR2 (M392T, K368M, K368E and C382R) and c-MET (N375S). Three somatic mutations in FGFR3: R248C, R252W and P698S were also observed although at very low frequencies, nevertheless, their presence suggests a possible role of FGFR3 mediated pathogenicity.

Beyond this, we also observed two mutations in KDR: Q 472H and L882F in KDR. The former, when observed in the germline context, is related to increased phosphorylation in VEGFR2 thus increasing the microvessel density in lung cancers (40). Three mutations were noted in A743V, R803Q and K860I in EGFR predicting a deleterious and damaging potential on the function of the protein. Single mutations were observed in TP53 (D208N) and STK11 (G58D) predicting deleterious and damaging potential. Single mutations in AKT1 (R48C) and FBXW7 (H470R) with unknown clinical significance were also noted.

Although most of these mutations are likely pathogenic, structurally and functionally damaging variants, as predicted by SIFT and Polyphen 2 programs, probability of a driver and passenger combination in these co-existing SNPs, needs to be understood in the context of Ewing's sarcoma pathogenesis.

Discussion

In the past decade, molecular targeted therapies have dramatically transformed the treatment landscape of breast, lung and colorectal cancer at various levels. To a greater extent, this has been contributed by the understanding of disease biology from the human genome project, advancements in genome sequencing and proteomic technologies to the clinical trial evaluations. Recurrent Ewing's tumors are chemo-resistant, with is no effective systemic therapy. Therefore, identification of mutations in genes for which therapeutic strategy exist can provide inputs for informed decisions to the development of novel drug combinations in this class of tumors. We therefore sought to identify novel mutations in recurrent Ewing's sarcoma that could provide leads to potentially identify therapeutic targets. Given the relatively high costs of whole genome sequencing, we performed a focused genetic analysis using 46 gene panel which consists of oncogenes, primarily kinases and tumor suppressors which are frequently mutated in most cancers.

The molecular mechanisms underlying onset and recurrence in Ewing's sarcoma pathogenesis is largely unknown. In an effort to decipher key somatic mutations which could have played a significant role in aggressive behavior of the tumor, we screened 789 hotspot mutations in 46 cancer related genes, which were primarily oncogenes and tumor suppressor genes (Ampliseq Panel v. 1.0) in 5 human Ewing's sarcoma tumor samples. One of the unique findings of this study, was the presence of a homozygous germ line polymorphism in APC gene (rs41115: GCC AC [G/A] GAA), which was earlier identified as a homozygous synonymous SNP in different population studies in sporadic colorectal cancer patients. Since there is no change in the protein sequence, this particular observation is most likely non pathogenic. However, the role of this genotype as a predisposition marker needs to be explored. We also observed a co-existing novel single nucleotide insertion in APC, (5, 112175668, T, TA: COSMIC 18965) that results in frame shift with elongation of the protein sequence (T1493fs*21) (c4477_4478 insA). So far, there has been only one report of this mutation in sporadic colorectal cancer (41-42). On similar lines, we observed another frame shift elongation mutation in TP53 gene (I254fs*10) (COSM43908), earlier reported in prostate cancer (43), is again novel in this study, Further

~~understanding on the functional consequences associated with insertions in APC and TP53 is warranted in correlation with gene expression studies, to ascertain its role on tumor onset and pathogenicity, in the context of Ewing's sarcoma. Some of these mutations could work as histology specific mutation signatures, which needs further validation. In addition, these signatures may have greater potential in addressing the diagnostic challenges in sub classification of the Ewing's disease.~~

~~Recurrence within 2 to 3 years of diagnosis in metastatic condition has been a great challenge in ES family of tumors with poor clinical outcome of less than 20%. The major factors associated with poor clinical outcome is the choice of therapy. So far, clinically proven molecular targeted therapies in Ewing's sarcoma is only towards IGF1R (16-20). We identified rare genetic changes that may have functional significance to the underlying biology and potential therapeutics for Ewing's sarcomas, which has never been reported before. The PI3K/AKT/mTOR signaling pathway is a central mediator driven by multiple signaling nodes such as RAS, EGFR, IGF 1R and c MET (44-45). Genomic profiling has revealed high prevalence of mutations in the PIK3CA/AKT/mTOR pathway in papillary breast carcinomas (46). Mutations in NRAS and PIK3CA, reflects alterations in PI3K/AKT and EGFR pathways, which could be potentially targeted by mTOR Inhibitors that are commercially available, such as Temsirolimus/everolimus. However, there have been reports which suggest co-existing RAS and PIK3CA activating mutations confers resistance to mTOR inhibitors in CRC (47), therefore adapting in Ewing's sarcoma requires further clinical trial evaluation before being implemented in routine practice. In another Phase I study on RAS mutated melanoma, MEK inhibitors selumatinib, trametinib, cobimetinib, pimasertib, and MEK 162, have shown promising results, which could be explored in Ewing sarcoma (48-52). Studies with dual MEK-ERK inhibitors have shown additive/synergistic effect with delayed emergence of acquired resistance to these inhibitors. In lung cancers, combination regimens for MEK inhibitors with either PI3K or AKT or STAT3 or BCL XL inhibitors has shown remarkable synergistic tumor regression, thus reducing the heterogeneity in response rates as compared to single drug regimens in mouse models with RAS mutation (52). The Q61R/P/K mutation has been invariably reported in melanoma and thyroid malignancies (53). The frequency of RAS mutations is high in pancreatic cancer (90%), colorectal cancer (40%), NSCLC (30%), bladder, peritoneum cancers (30%), cholangiocarcinoma and melanoma (15%). In hematolymphoid malignancies and sarcomas, the RAS mutations are rarely observed. Therefore, extending this study to a larger subset of patients with recurrent~~

Ewing's Sarcoma could shed some light on the role of RAS mutations and their therapeutic value.

In the early embryonic stage of the fetus, the normal organ development, vascularization and skeletal development is driven primarily by FGFR family of receptor and their interacting partners in the signaling pathway. However, genetic alterations/over expression of FGFR has been shown to play a key role in proliferation and angiogenesis in several tumors.

It has been well known that mutations in FGFR family (FGFR1-3) of receptors are associated with syndromic craniosynostosis and short limb dwarfism when expressed in autosomal dominant form. The last decade, several studies have been reported on the role of FGFR1-3 activation in human cancers, thus suggesting its potential therapeutic value. We observed: M392T, C383R, K368M, K368E in FGFR2, with C383R being common in all the cases. Although the functional role of these mutations predicts a benign phenotype, there are some clinical studies on endometrial carcinomas where C383R mutation in FGFR2 is associated with poor prognosis (54). Mutation screening for oncogenic activation in squamous carcinoma of lung, uterine, cervical and bladder cancers has revealed the presence of FGFR2/3 activating mutations, indicating a common functional role in epithelial malignancies (55). We also observed a common SNP in all the five cases in PDGFRA; V824V (COSM22413). This mutation, although silent, is commonly observed in soft tissue, CNS and endometrial tumors.

Some of the mutations observed here, are already used as therapeutic markers for evaluating treatment planning in other malignancies. Therefore, further clinical validation of our findings in a larger cohort of Ewing's Sarcoma could help in deriving novel therapeutic options for these patients. To summarize, the findings from this pilot study could also help in deriving unique genomic signatures for molecular classification of tumors beyond their relevance as promising drug targets in four major pathways such as EGFR, PI3K/AKT/mTOR, FGFR/IGF-1R and c-MET towards improving clinical outcomes in Ewing's sarcoma. These pathways are also affected in most of the other cancers.

In conclusion, this pilot study of targeted sequencing in ES, revealed presence of somatic mutations in tumor suppressors and oncogenes with commercially available inhibitors/inhibitors in clinical trials. While the former has a potential role in tumorigenesis, the latter indicates possibilities of novel therapeutic options. Last, but by no means least, much still needs to be done to improve clinical outcome in Ewing's Sarcoma. Further experimental and clinical validation of these markers is warranted in order to establish these genes as important biomarkers or targets

~~in Ewing's sarcoma. We hope that this pilot study of genotyping in 5 recurrent cases of Ewing's sarcoma ignites interest for further investigations to understand the molecular pathology of this disease. Also, study findings such as ours; suggest the clinical utility of performing a genotype directed clinical trial to test novel drug combinations in these patients.~~