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A Study of EWSR1Gene Rearrangement by Fluorescence In-Situ Hybridization in Ewing's Sarcoma Patients

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Received Date: 07 February 2024 **Accepted Date**: 23 February 2024 **Published Date**: 04 March 2024.

Citation: Dharmesh M. Patel, Dhrumil Parmar, Pina J. Trivedi, Mahnaz M. Kazi, et al, (2024), A Study of EWSR1Gene Rearrangement by Fluorescence In-Situ Hybridization in Ewing's Sarcoma Patients, *Clinical Research and Clinical Reports*, 3(2); **DOI:**10.31579/2835-8325/064

Abstract

Introduction: Ewing's sarcoma is a rare form of cancer affecting bones or the adjacent soft tissues, primarily seen in individuals aged between 10 to 30 years. As the second most common malignant bone tumor in this age group, it constitutes around 6% to 10% of all malignant bone tumors. Notably, its identification is linked to the EWSR1 gene translocation, making Fluorescence In-Situ Hybridization (FISH) analysis pivotal for accurate diagnosis. Objective of the present study to scrutinize the correlation between the EWSR1 gene translocation and the clinicopathological characteristics in patients diagnosed with Ewing's sarcoma. This research aims to investigate EWSR1 gene translocation through the Fluorescence In-Situ Hybridization (FISH) technique. This approach holds significance as it involves mastering FISH techniques, assessing the link between EWSR1 gene translocation, and exploring how it relates to various clinicopathological aspects in patients with Ewing's Sarcoma.

Method: Histopathologically confirmed patients with Ewing's sarcoma were included. EWSR1 gene rearrangement was detected in total 35 patients with Ewing's sarcoma tissue using Fluorescence In-Situ Hybridization. FISH was performed using EWSR1 Dual Color Break Apart probe kit.

Result: 35 patients were enrolled in the study, among them 60% (21/35) of the patients were male and 40% (14/35) of the patients were female and the median age was 16 years. EWSR1 gene rearrangement was positive in 71% (25/35) of the patients while 29% (10/35) of the patients were negative. EWSR1 gene rearrangement was significantly associated with site and nearly significant with lymph node involvement but not with age, gender, size, lung metastasis, and not significant association with histopathological parameters.

Summary and Conclusion: This study illustrates the importance of FISH as an ancillary diagnostic tool in the diagnosis of EWSR1 rearranged neoplasms. EWSR1 gene rearrangement is significantly higher in soft tissue followed by bone and parenchymal organs. Present study shows positive for EWSR1 rearrangement by FISH technique in 2 (9%) CD99 negative patients, 6 were weak positive patients and 3 (14%) were weakly positive for FLI1 by Immunihistochemistry (IHC) this signifies the role of molecular studies in cases difficult to diagnose on routine histopathology and IHC.

Key words: laser; patients

Introduction

Ewing's Sarcoma can occur at any age but, the peak incidence occurs in the second decade of life, and males suffer from ES about one and half times more often than females. (Javed MU et. al.; 2009) Generally, ES can occur at any part of the body but, most commonly affects bone particularly pelvis, femur or axial skeleton and, it can also involve soft tissues in upto 30% of patients. **(Applebaum MA. et. al.; 2011), (Ordonez JL. et. al.; 2009), (Kauer M. et. al.; 2009)** However, the diagnosis is reached in most cases only with the help of IHC. On the other hand, some cases require molecular studies for the confirmation of the diagnosis. (Gambheri G. et. al.; 2011) Gene rearrangement resulting in a translocation are a defining diagnostic feature in many hematopoietic and solid tumors. Detection of specific gene rearrangement by Fluorescence In-Situ Hybridization (FISH) is commonly used in practice of pathology, in addition to immunohistochemistry, to aid in the diagnosis of more difficult cases. Moreover, FISH method is found to be more sensitive and specific compared to Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR) on Formalin Fixed Paraffin Embedded (FFPE) tissue section and consists of 91% of sensitivity and 100% specificity. Therefore, it is easier to interpret the results. **(Bridge RS. Et. al.; 2006)** Ewing sarcoma breakpoint region 1 (EWSR1) gene is located at 22q12 translocation involving the EWSR1 gene was first described in and first to molecularly defined Ewing's Sarcoma. (Delattre O. et. al.; 1994) The majority of Ewing's Sarcoma family of tumors is defined by a translocation resulting in the fusion of the EWSR1 gene and the gene of the E26 transcription-specific (ETS) family of transcriptors factor such as FLI1, ERG, ETV1, E1AF and FEV. hotmail.com (Cantile M. et. al.; 2013) The aim of this research is to study EWSR1 gene translocation by Fluorescence In-

Situ Hybridization (FISH) technique which is significant because it involves learning FISH technique, evaluating the association of EWSR1 gene translocation using FISH technique and correlating EWSR1 gene translocation with various clinicopathological characteristics of Ewing's Sarcoma patients.

Materials and Method

Patient Details: This retrospective study, sanctioned by the institutional scientific and ethics committee, encompassed 35 cases of Ewing's Sarcoma patients diagnosed morphologically and immunohistochemically at The Gujarat Cancer & Research Institute (GCRI). All cases involved excision specimens, with exclusions made for those with inconclusive diagnosis of ES and specimens lacking proper formalin fixation resulting in cellular detail loss. Macroscopic examination of the specimens adhered to established guidelines, followed by staining with Hematoxylin and Eosin (H&E) and a panel of immunohistochemical stains including vimentin, MIC-2 (CD99), TdT, synaptophysin, FLI1, and desmin immunostain. Pertinent clinicopathological data such as age, gender, tumor size, histopathological type, stage, and metastasis were retrieved from the maintained case files in the Medical Record Department of the institute. Disease status evaluation was conducted through clinical examination, radiological investigations, and biochemical assessments. (Table 1)

CHARACTERS	N(%)
Age (Median: 16 years, Range: 1-60)	
≤16 years	18(51)
>16 years	17(49)
Gender	
Male	21(60)
Female	14(40)
Tumor size	
≤3cm	25(86)
3.1-8cm	02(07)
>8cm	02(07)
Site	
Bone	21(60)
Soft tissue	10(29)
Parenchymal organs	04(11)
Lymph node	
Negative	25(71)
Positive	10(29)
Lung metastasis	
Negative	33(94)
Positive	02(06)
Stage	
I	18(56)
IV	14(44)
FLI1	
Negative	02(09)
Weak positive	03(14)
Positive	17(77)
CD99	
Weak positive	09(26)
Positive	26(74)
Desmin immunostain	
Negative	35(100)
Vimentin	
Negative	03(21)
Weak positive	04(29)
Positive	07(50)
Synaptophysin	
Negative	11(73)
Weak positive	02(13)
Positive	02(13)
TdT	
Negative	35(100)

Table 1: Clinical characteristic of the EWSR1 gene rearrangement patients

Fluorescence In-Situ Hybridization (FISH) technique: FISH analysis was conducted on paraffin blocks using the LSI EWSR1 Dual Color Break-Apart probe (Zytovision). Tumor tissue sections, 4 µm thick, were utilized for the FISH studies on coated slides. Post- deparaffination, a series of washes involving xylene, alcohol, and TDW were administered. Slides underwent Pre-treatment solution treatment at 95°C for 25 minutes, followed by pepsin

treatment at room temperature for 20 minutes. For the hybridization process, 1 µl of the probe was applied to the target area, covered with a coverslip, sealed with rubber cement, and then incubated at 37°C overnight. The subsequent day, the coverslip was removed, and the slide underwent post-hybridization washes, with 1 drop of DAPI counterstain applied before placing the coverslip. Observation of the slide was carried out using an Epi-fluorescence microscope, and scoring and capture were performed using Isis software.

Statistical analysis

The statistical analysis in this study was conducted using SPSS statistical software, version 26, (developed by SPSS Inc., U.S.). Key descriptive statistics, including mean values, standard error of the mean (SE), and median values, were computed to summarize the data. To examine the relationship and significance between two variables, Pearson's Chi-square ($\chi 2$) test and Pearson's correlation coefficient (r) were employed as statistical tools. In accordance with common statistical conventions, P values less than or equal to 0.05 were deemed indicative of statistically significant findings.

Result

Out of total 35 (100%) patients, 10 (29%) patients showed 2F signal pattern. Hence, these patients were considered negative for EWSR1 rearrangement. While 25 (71%) patients showed 1O1G1F (OGF) signal pattern considered positive for EWSR1 rearrangement (Figure 2).

Figure 2 Representative images of FISH results for EWSR1 gene rearrangement

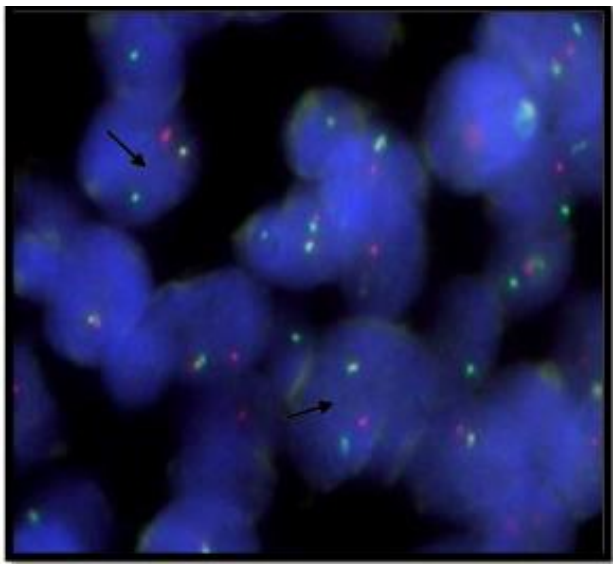

Figure 2A: Positive result for EWSR1 gene rearrangement

Image shows OGY pattern describing 1O1G1F signal shows positive result for EWSR1 gene rearrangement.

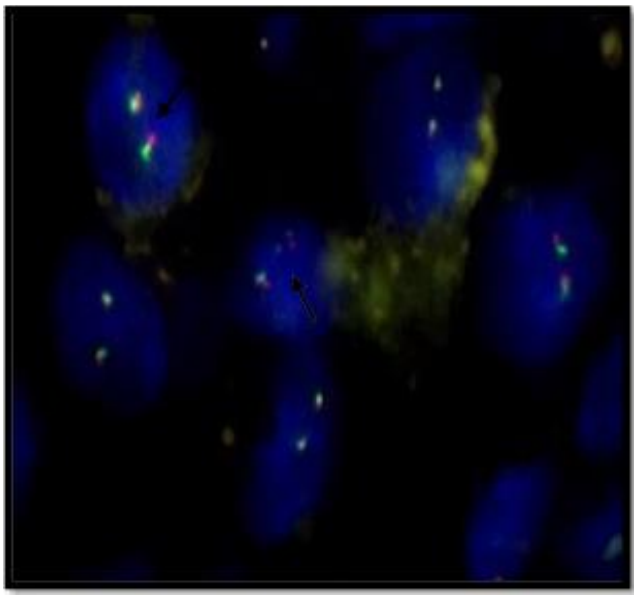

Figure 2B: Negative result for EWSR1 gene rearrangement

Image shows YY pattern describing 2F signal shows negative result for EWSR1 gene rearrangement.

Among the total enrolled patients, 71% (25/35) patients were positive for EWSR rearrangement, while 29% (10/35) were negative. Out of total patients 60% (21/35) were male, among that 67% (14/21) exhibiting positive EWSR1 rearrangement and 33% (7/21) showing negative results. Additionally, among the 14 female patients, 79% (11/14) were positive for EWSR1 rearrangement, while 21% (3/14) were negative. $\chi 2$=0.583, r = -0.129 and p= 0.460. (Table 2)

Parameter	Incidence of EWSR1 gene rearrangement			χ^2	r	p
	Total N=35	Positive N=25	Negative N=10			
	N (%)	N (%)	N (%)			
Gender	**35**	**25**	**10**			
Male	21 (60)	14 (67)	07 (33)			
Female	14 (40)	11 (79)	03 (21)	0.583	- 0.129	0.460
Age	**35**	**25**	**10**			
≤16 years	18 (51)	13 (72)	05 (28)			
>16 years	17 (49)	12 (71)	05 (29)	0.011	0.018	0.918
Size	**29**	**20**	**09**			
≤ 3 cm	25 (71)	16 (64)	09 (36)			
3.1-8 cm	02 (06)	02 (100)	00 (00)	1.291	0.009	0.963
> 8 cm	02 (06)	01 (50)	01 (50)			
Site	**35**	**25**	**10**			
Bone	21 (60)	16 (76)	05 (24)			
Soft tissue	10 (29)	08 (80)	02 (20)	7.560	-0.421	**0.012**
Parenchymal organ	04 (11)	01 (25)	03 (75)			
Lymph node	**35**	**25**	**10**			
Absent	25 (71)	20 (80)	05 (20)	3.150	-0.300	**0.080**
present	10 (29)	05 (50)	05 (50)			
Lung metastasis	**35**	**25**	**10**			
Absent	33 (94)	24 (72)	09 (27)	0.477	-0.177	0.504
Present	02 (06)	01 (50)	01 (50)			
Stage	**29**	**19**	**10**			
I	19 (66)	14 (74)	05 (26)	1.627	-0.237	0.216
IV	10 (34)	05 (50)	05 (50)			

Table 2: correlation of EWSR1 gene with clinicopathological parameters

From the entire patient, 51% (18/35) patients were 16 years or younger, among that 72% (13/18) were positive for EWSR1 rearrangement and 28% (5/18) were for negative results. Furthermore, within the group, 49% (17/35) were female, of which 71% (12/17) tested positive and 29% (5/17) were negative for EWSR1 rearrangement. $\chi 2$=0.011, r= 0.018 and p=0.918. (Table:2)

The study encompassed three primary sites: Bone (60%), Soft tissue (29%), and Parenchymal organs (11%). Among the patients with bone involvement, 76% (16/21) tested positive, while 24% (5/21) tested negative for rearrangement. (Figure 1)

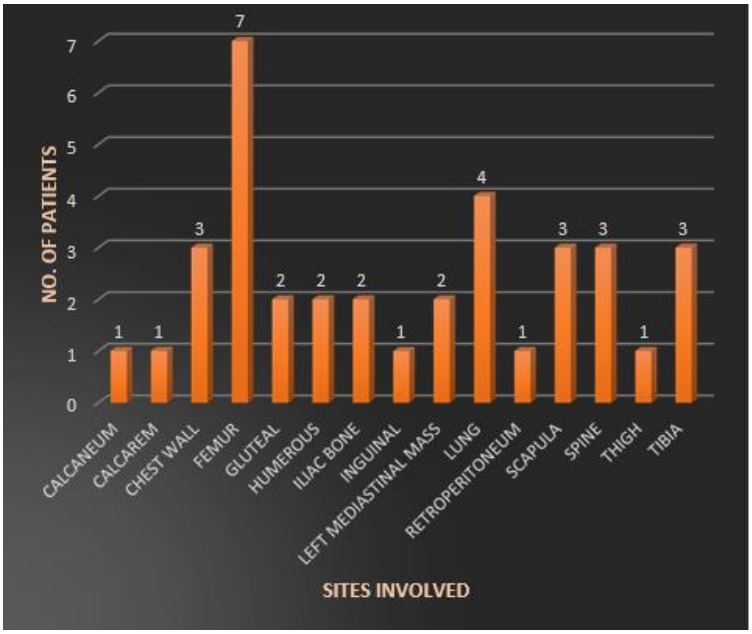

Figure 1: different sites involved in Ewing's Sarcoma

In the case of soft tissue, 80% (8/10) of patients showed a positive result, and 20% (2/10) displayed a negative outcome. Furthermore, among patients with parenchymal organ involvement, 25% (1/4) tested positive, while 75% (3/4) tested negative for EWSR1 rearrangement. Notably, the study revealed a highly significant association between the specific sites and the EWSR1 gene. $\chi 2=7.560$, r= - 0.421, p=0.012. (Table 2, Figure 3)

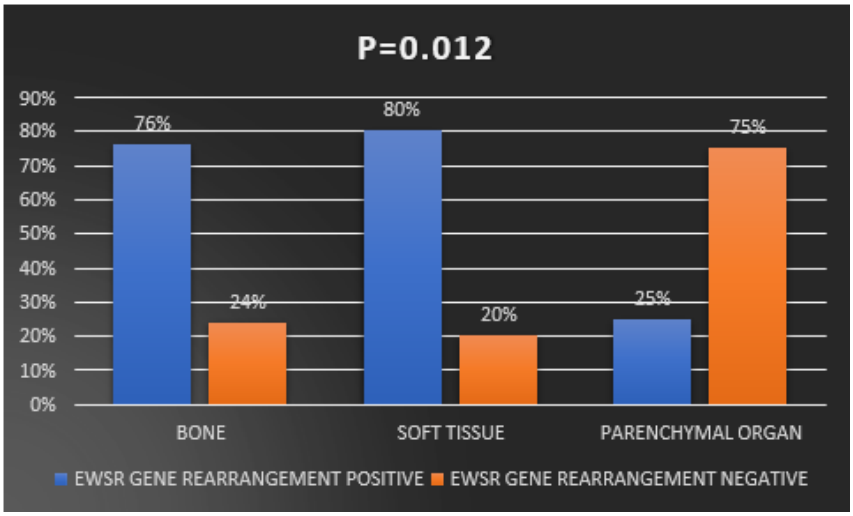

Figure 3: Correlation between EWSR1 gene rearrangement with sites

Lymph node involvement was detected in 29% (10/35) of the patients, with 50% (5/10) testing positive and 50% (5/10) testing negative for EWSR1 gene rearrangement. Conversely, 71% (25/35) of the patients did not show lymph node involvement, with 80% (20/25) testing positive and 20% (5/25) testing negative for EWSR1 gene rearrangement. This study indicated a near significance between lymph node involvement and EWSR1 gene rearrangement. $\chi 2= 3.150$, r= -0.300, p= 0.080. (Table 2, Figure 4)

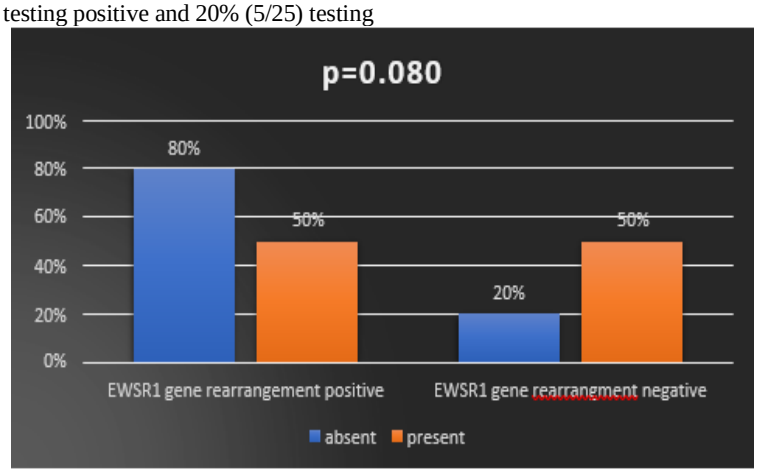

Figure 4: Correlation between EWSR1 gene rearrangement with lymph node metastasis

Ewing's sarcoma is a highly aggressive cancer comprised of undifferentiated round cells. Its

Discussion

Ewing's sarcoma is a highly aggressive cancer comprised of undifferentiated round cells. Its

exact origin is uncertain. This cancer typically affects individuals between the ages of 10 and 30, and it is known for its rapid growth and tendency to spread to other parts of the body. **(Lin PP. et. al.; 2011)** Ewing's sarcoma exhibits a peak incidence during the second decade of life,

with a notable predilection for males, affecting them at a rate approximately one and a half times higher than females. **(Riggi N. et. al.; 2007)** Ewing's sarcoma typically presents a predilection for diverse anatomical sites within

the body. While it may manifest in virtually any location, it most frequently afflicts the skeletal system, with a notable predilection for the femur, pelvis, scapula, and the axial skeleton. Nevertheless, this malignancy also demonstrates an ability to infiltrate soft tissues and parenchymal organs in affected patients,

underscoring its capacity for anatomical versatility. **(Applebaum MA. et. al.; 2011)**

Ewing Sarcoma, denoted as ESFT (Ewing Sarcoma Family of Tumors), is distinguished by non- random chromosomal translocations, which culminate in the formation of fusion genes

positioned at the C-terminus of the transcribed region. These fusion proteins harbour a distinctive feature, namely the presence of RGG repeats that facilitate their binding to RNA molecules, thereby actively engaging in transcriptional processing and RNA splicing. The N-terminus of the fusion protein originates from the ETS family of transcription factors, with members such as FLI1, ERG, FEV, and others playing a pivotal role in this context. Notably, the t (11;22) (q24; q12) translocation is a hallmark event, observed in 85-90% of Ewing Sarcoma tumors, effectively generating the fusion protein. The remaining 10- 15% of cases, on the other hand, entail alternative translocations such as t (7;22) (p22; q12) and other less common chromosomal rearrangements. **(Riggi N. et. al.; 2007)**

The identification of EWSR1 gene rearrangements can be accomplished through FISH utilizing

Break-Apart probes on FFPE tissue specimens. FISH stands out as a prominent and extensively employed technique for molecularly diagnosing

ESFT due to its ability to be executed with minimal tissue samples and its compatibility with FFPE materials. This method is increasingly gaining prevalence in numerous medical centers as a pivotal tool for confirming the molecular diagnosis of ESFT. **(Murthy SS. et. al.; 2021)** FISH offers two distinct approaches for analysis, namely the fusion technique and the break-apart technique, both of which consistently yield congruent results. The choice to employ the FISH break-apart strategy is typically made when multiple translocation partners are involved in the genetic rearrangement under investigation. This strategy proves to be a cost-effective alternative, marked by its capacity to generate brighter and larger signal patterns, rendering interpretation of the results a more straightforward and efficient process. **(Bridge RS. et. al.; 2006)**

In the present investigation, a rearrangement of the EWSR1 gene was detected in 71% (25/35) of the patients, consistent with the findings of Murthy S. et al. (2021) who reported a 79% incidence, and Bashir M. et al. (2020) who identified an 88% occurrence of the EWSR gene rearrangement. Various published studies have documented the presence of EWSR1 gene rearranged tumors in the diagnosis of ESFT, with reported rates of 92% (144/156) **(Gambheri G. et. al., 2011),** 82% (89/109) **(Noujaim J. et al.; 2017),** 91% in a series with no false positives **(Bridge RS. et al.; 2006)**, and 83% (15/18) of cases **(Qian X. et al.; 2005).** Indian studies have also reported EWSR1 positive outcomes via FISH in a limited sample size, with rates of 91% (10/11) (Jambhekar NA et al.; 2006) and 92% (12/13) of cases **(Rekhi B. et al.; 2014).**

The high incidence of EWSR1 gene rearrangement was found in soft tissue for about 80% of the patients. The study found significant correlation between sites with EWSR1 gene rearrangement. (p=0.012) Similar studies was done by Bashir M. et. al. study and they found 51% had bone involvement. **(Bashir MR. et. al.; 2020)**

To conclude, FISH emerges as a robust and dependable adjunctive technique in the diagnostic armamentarium for identifying Ewing's sarcoma and pinpointing the translocation partner genes involved. This investigation underscores the pivotal role of FISH as a supplementary diagnostic tool in the evaluation of neoplasms featuring EWSR1 gene rearrangements. Notably, the prevalence of EWSR1 gene rearrangements is notably higher in soft tissue tumors, followed by occurrences in bone and parenchymal organs. Within our study cohort of 25 patients, FISH analysis identified 6 patients with weak CD99 positivity, while 2 patients (comprising 9%) exhibited a lack of CD99 expression, and 3 patients (14%) showed only weak FLI1 positivity by IHC. This underscores the invaluable contribution of molecular studies in cases that present diagnostic challenges through routine histopathological and IHC analysis.

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