Sarcoma Research - International

Open Access

Austin Publishing Group

Research Article

Study of EWSR1 Dual Colored Probe FISH Assay on Tumors and Tissues not Known to Have EWSR1 Translocation on Paraffin Sections: Potential Pitfalls in Interpretation of FISH Signal Separation

Sadri N[1], Puthiyaveettil R[2] and Zhang PJ*[2]

[1]Department of Pathology and Laboratory Medicine, University Hospitals, Cleveland, OH, USA

[2]Department of Pathology and Laboratory Medicine, Hospital of the University of Pennsylvania, Philadelphia, PA, USA

***Corresponding author:** Paul J Zhang, Department of Pathology and Laboratory Medicine, Hospital of the University of Pennsylvania, Philadelphia, PA, USA

Received: August 16, 2016; **Accepted:** November 21, 2016; **Published:** November 23, 2016

Abstract

Two colored DNA probes flanking the specific break point of interest, when hybridized can visualize the probe signal separation in tumor cells with translocation of the gene of interest. Due to its commercialization, this breakapart FISH assay has been used widely on routine surgical specimen for diagnostic purpose. Currently there is lack of standardization on the analytical criteria. Different distances of signal separation and methodology to calculate the % of cells have been used in literatures. The specificity of breakapart probe on tumors known to have unrelated translocation or other genetic abnormality is unknown. We evaluated most commonly used *EWSR1* breakapart probes (Vysis) on 11 sarcomas known to have gene translocation other than *EWSR1*, 6 breast carcinomas with Her2 amplification and 2 normal tonsils. For control, 5 tumors known to have *EWSR1* translocation were also evaluated. Range of signal separation can be seen in all non-*EWSR1* related tumors and normal tonsillar lymphocytes. Our analysis suggests that previous published thresholds based on false-positive in wild-type cells may not fully reflect the greater variation seen in tumor cells. We also found that aneuploid *EWSR1* was very common in sarcomas with non-*EWSR1* translocation but not in *EWSR1* related tumors, the significance of which is unknown. The observed signal separation by FISH in tissue known to negative for the translocation is likely an artifact related to the paraffin tissue process and FISH assay procedures and less likely due to genetic instability of the tumor cells as it also occurs in tonsillar lymphocytes. In addition to focus on the width of signal separation and % of tumor cells with signal separation, evaluation of tumor cells with normal allele signals might be another helpful pitfall to present false positivity in FISH breakapart signal analysis.

Keywords: EWSR1; FISH; Sarcoma; Break-apart

Introduction

Gene rearrangements resulting in a translocation are a defining diagnostic feature in many hematopoietic and solid tumors. Detection of specific gene rearrangements 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. The FISH probe design more frequently used for this purpose is the breakapart probe design in which the dual colored probes flanking the break point of the gene [1]. When a translocation takes place, the two normally fused colored signals will appear separated (break apart) in the tumor cell nuclei. The relative distance between the differentially colored probes of a signal pair within the nucleus is visually estimated as the basis of determining presence of translocation or not. As such, the key determinant in accurate interpretation of the breakapart assay is to determine the thresholds for the width of signal separation and frequency of these events that should be considered significant to indicate a true translocation. However, a perceived signal separation in variety of cells known to have no translocation (wild type) as a result of artifacts created by tissue processing and its impact to FISH breakapart assay analysis have not been evaluated.

Ewing's sarcoma breakpoint region 1 (*EWSR1*) gene is located at 22q12. Translocation involving the *EWSR1* gene was first described in and first to molecularly define Ewing's sarcoma [2]. The majority of Ewing's sarcoma family of tumors is defined by a translocation resulting in the fusion of the *EWSR1* gene and a gene of the E26 Transformation-Specific (ETS) family of transcription factors such as *FLI1, ERG, ETV1, E1AF*, and *FEV* [2-4]. However, other members of the TET family such as FUS and transcription factors other than ETS family such as NFATc2 could substitute *EWSR1* and ETS family member in translocation of rare tumors of Ewing's sarcoma family [3-6]. On the other hand, the rearrangement of the *EWSR1* gene is not specific to the Ewing's sarcoma family of tumors and can be seen in a broad range of malignant and benign mesenchymal and non-mesenchymal neoplasms distinct translocations involving the *EWSR1* gene and non-ETS transcription family members are seen in several other mesenchymal neoplasms, including desmoplastic small-round-

Citation: Sadri N, Puthiyaveettil R and Zhang PJ. Study of *EWSR1* Dual Colored Probe FISH Assay on Tumors and Tissues not Known to Have *EWSR1* Translocation on Paraffin Sections: Potential Pitfalls in Interpretation of FISH Signal Separation. Sarcoma Res Int. 2016; 3(3): 1034.

cell tumor, clear cell sarcoma of soft tissue, extra skeletal myxoid chondrosarcoma, soft tissue myoepithelioma and angiomatoid fibrohistiocytoma [4,7]. In non-mesenchymal tumors, *EWSR1* gene fusions have also been described in hyalinizing clear cell carcinoma of salivary gland) and mucoepidermoid carcinoma of salivary gland [8,9].

FISH analysis for *EWSR1* gene rearrangement using commercially available breakapart probes allows for a reasonable ancillary test to identify the expanding numbers of *EWSR1*-rearranged tumors and is one of the most commonly used FISH assays for diagnosis in solid tumor pathology. Cancer genomes are complex with many small and large scale genomic events. No study has previously looked at how the *EWSR1* breakapart FISH assay performs in *EWSR1* wild type tumors. We evaluated *EWSR1* breakapart probe signals in series of epithelial and mesenchymal tumors to investigate the distance and frequency of signal separation in *EWSR1*wild type tumors in contrast to *EWSR1* translocation positive tumors.

Materials and Methods

Samples

The study was performed as an internal QA project in the FISH lab at Anatomic Pathology, Hospital of the University of Pennsylvania. For the *EWSR1* wild type tumors, we chose 11 mesenchymal tumors with known non-*EWSR1* translocation: five synovial sarcomas (with SYT translocation), five alveolar rhabdomyosarcomas (FKHR translocation), and one sclerosing epithelioid fibrosarcoma (SEF, FUS translocation); and six non-mesenchymal tumor samples (six breast cancer samples with positive HER2 amplification) and two normal tonsil samples. Five *EWSR1* translocation tumors (2 Ewings sarcoma, 1 PNET, 1 extraskeletal myxoid chondrosarcoma, 1 desmoplastic small round cell tumor) were included for comparison. All samples processed by standard FFPE methods. Briefly, the specimens were received fresh, fixed in 10 % buffered formalin for less than 12 hours, and processed for routine histological analyses. FISH analyses were done on 5-µm-thick sections.

FISH

Fluorescent in Situ Hybridization (FISH) for rearrangement of the *EWSR1* locus was performed using a *EWSR1*dual-color breakapart probe (22q12) (Vysis/Abbot Laboratories, Downers Grove, IL). The 1100 kb probe specific for the 3' telomeric side of EWS was labeled in Spectrum Green and the 500 kb probe specific for the 5' centromeric side of EWS was labeled in Spectrum Orange. These FISH assay was performed according to manufacturers' instructions with some modifications. In brief, 4-5 micron sections were mounted on plus-charged slides and baked overnight at 56°C. Slides were deparaffinized in CitriSolv (Fisher, Vernon Hills, IL), dehydrated in 100% ethanol and air dried. Slides were pretreated in 0.2 NHcl for 20 minutes and then with the pretreatment reagent (Abbott Molecular, IL) for 30 minutes at 80°C. After the pretreatment, the slides were digested in 0.5mg/ml pepsin solution at 37°C for 14-15 minutes. After digestion, slides were post-fixed in 10% buffered formalin and dehydrated in 85% and 100% ethanol and dried on a slide warmer at 45-50°C for 3 minutes. 10µl of the probe mixture was applied to the target area on the slide and covered with a cover-slip sealed with rubber cement. The slides were then placed in the Thermobrite system (Abbott Molecular, IL) for denaturing at 80°C for five minutes and hybridization at 37°C for overnight. After overnight hybridization, cover slip was removed and the slides were immersed in pre-warmed post-hybridization wash buffer (2x SSC/0.3% NP40, ph 7.2) at 72°C for 2 minutes, then air dried in the dark. 10µl of DAPI 1 solution (Abbott Molecular, IL) were applied to the hybridized area and cover slipped and sealed. A fluorescent scope (DM 5000 B, Leica) equipped with a FISH Imaging System Isis V5.4.7 (Metasystem) was used to analyze the FISH signals by the two co-investigators (PJZ and NS). A live review of each case was performed first with a triple pass filter (Dapi, Spectrum Green and Spectrum Orange) under X40 and X60 lenses and representative areas were captured in images by Isis system for later signal analysis.

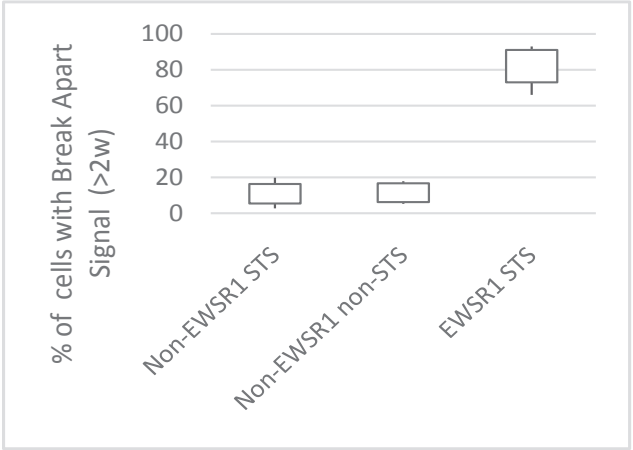

Figure 1: Percentage of cells with ≥2SW signals in each group.

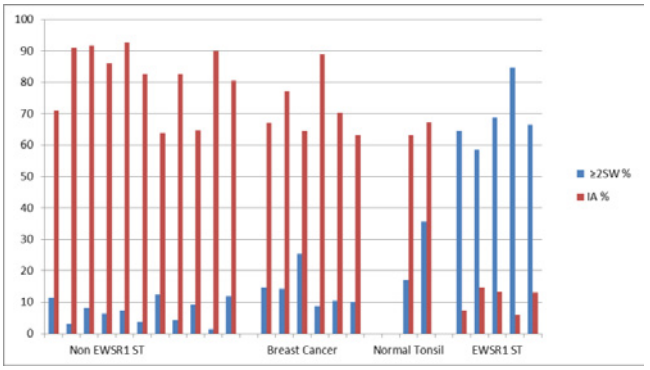

Figure 2: Percentage of tumor cells with ≥2SW and IA in each case.

Analysis

At least 100 cells were counted (median: 145, average: 160) unless less than 100 cells were available to count because of the specimen size. Cells with no signal, only one unpaired signal or one intact allele (fused signals), or cells with signals in the overlapped nuclei were considered non-informative and not analyzed to avoid truncation or overlapping artifact. Cells included in analysis were morphologically neoplastic (except tonsils) with no or minimal nuclear overlapping. Tumor cells passing this parameters were categorized to have:1, intact alleles (IA, at least two), 2, at least one of two alleles with a pair of separated signals, or 3, intact allele(s) with Unpaired Orphan Signal(s) (UPS); respectively. The relative distance between the differentially colored probes was visually estimated live and on captured images

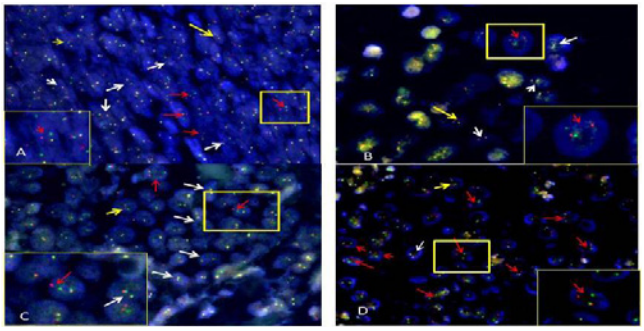

Figure 3: Representative images of FISH *EWSR1* breakapart signals in (A) synovial sarcoma; (B) alveolar rhabdomyosarcoma; (C): ductal breast carcinoma; and (D) Ewing's sarcoma. Examples of cells with two intact alleles (white arrow), or with 1 Signal Width (SW) breakapart signal and one intact allele (yellow arrow), or cells with ≥2SW breakapart signals and one intact allele (red arrow) are highlighted. Original magnification 400x, insert: close up of yellow square.

and categorized as 1 Signal Width (SW), 2 SW, 3 SW, 4 SW or > radius of the nucleus. The average *EWSR1* alleles per cell were also determined in each case. The frequency (%) of cells containing at least 2SW signal separation was calculated as the number of cells with ≥2SW signal divided by as the total number of cells with Intact Alleles (IA) plus the number of cells with signal ≥2SW. Cells with unpaired orphan signal(s) were not included in the analysis to avoid truncation artifact.

Results

We observed a similar frequency of 1SW breakapart events in *EWSR1*-related tumors group (9±3.4%) and non-*EWSR1* group (11.3±2.9%). As such we used a minimal 2 SW threshold to characterize the signal separations observed. As expected, the *EWSR1*- tumors showed a positive breakapart signal (≥2SW) in high percentage of cells (82.2±10.5%) (Figure 1). Surprisingly, similar 'positive' breakapart signals (≥2SW) were observed in non-*EWSR1* associated tumors, although at a much lower frequency (10.9±5.2%). Cells with two Intact EWRS1 Alleles (IA) were seen in a far lower frequency in *EWSR1*-related group (10.8±3.9%) as compared to non-*EWSR1* associated tumors (78.1±11.1%) (Figure 2,3). Aneuploid *EWSR1* (>2.0 *EWSR1* alleles in cell) was commonly seen in non-*EWSR1* sarcomas (3.5±1.1 *EWSR1* alleles per cell) but was not in the *EWSR1*-related group (Table 1).

Table 1: *EWSR1* allele per cells.

Group	n	EWSR1/cell (mean)
Non EWSR1 ST	11	2 to 5 (3.5)
EWSR1+ Tumor	5	2
Breast Caner	6	2-2.5 (2.3)
Tonsil	2	2

Discussion

When validating the FISH assay, each laboratory needs to identify and establish thresholds for determining criteria for break apart. Prior studies have suggested that cutoff for positive *EWSR1* breakapart result in small blue round cell tumors to be >15%-20% of cells with >1SW break [10,11]. With discovery of *EWSR1* rearrangements in an increasing number of tumors, it is important to characterize the *EWSR1* breakapart signal in tumors known to be *EWSR1* wild type. To our knowledge no prior reported study has focused on *EWSR1* breakapart signal distribution in non-*EWSR1* sarcomas or other cancer types with wild type *EWSR1*.

In this study, we focused on other sarcomas with confirmed other driver gene rearrangement and conventional ductal carcinoma of the breast to limit the possibility of a true *EWSR1* translocation in our 'non-*EWSR1*' (*EWSR1* wild type) cohort. Small separation of probe signals (<2SW) was seen at equal frequency in all tumor cells tested and probably represents a non-specific change. Surprisingly, wider probe signal separation (>2SW) was seen in all cases tested up to more than 20% of tumor cells expected not to have an *EWSR1* translocation. Similar wide signal separation was also seen in normal tonsillar lymphocytes up to more than 30% of the cells. The exact cause of this signal separation is unclear but likely related to certain degree of chromosome/DNA elasticity under the impact of various paraffin tissue processing and FISH procedures. Alternatively, it might represent an *EWSR1* instability in minor population of tumor cells. Nevertheless, this signal variation should be regarded as nonspecific noise background rather than specifically representing *EWSR1* translocation for diagnosis. Although probes for detecting other translocations were not evaluated, as the factors attributed to the non-specific *EWSR1* signal breakapart likely exist in the paraffin tissue and the FISH protocol, a conservative approach should be applied to interpret signal separation when using other breakapart FISH probes. In contrast, in tumor with *EWSR1* translocation, not all tumor cells show wild separation of the FISH signals. Seeing small percentage of tumor cells with clear normal IA signals (<20%) should not be used to rule out presence of translocation. However, if more than 50% of the clearly defined tumor cells show normal IA signals, diagnosis of translocation should be more cautious. Interestingly, non-*EWSR1* associated tumors commonly harbor aneuploidy *EWSR1* (IA>2.0) but the significance of this observation is unknown and likely related to the overall aneuploid status of these tumors.

In summary, our findings implicate that widely separated signals by breakapart FISH assay in small number can be seen in tumors or tissue not known to have EWRS1 translocation as diagnostic hallmark and therefore it is nonspecific for translocation. Our analysis suggests that previous published thresholds based on false-positive in wild-type cells may not fully reflect the greater variation seen in tumor cells. As it is widely accepted that the diagnostic threshold be calculated as three standard deviations from the mean of false-positive findings in wild-type cells, our analysis suggests that cutoff for positive *EWSR1* FISH breakapart assay should be that >25% of tumor cells (3 standard deviation from the mean of noise background) show wide separation breakapart signal (defined as least 2SW). In tumors with positive translocation, small number of tumor cells might show normal wild type *EWSR1* signals. However, if a tumor shows more cells with IA (wild type) signals than breakapart (≥2SW) signals, caution should be excised to prevent false positive result.

References

1. Lee W, Han K, Harris CP, Shim S, Kim S, Meisner LF. Use of FISH to detect chromosomal translocations and deletions: analysis of chromosomal rearrangement in synovial sarcoma cells from paraffin-embedded specimens. Am J Pathol. 1993; 143: 15–19.

2. Delattre O, Zucman J, Melot T, Garau XS, Zucker JM, Lenoir GM, et al. "The Ewing Family of Tumors--a Subgroup of Small-Round-Cell Tumors Defined by Specific Chimeric Transcripts." *The New England Journal of Medicine*. 1994; 331: 294–299.

3. Tony LNg, O'Sullivan, Pallen CJ, Malcolm Hayes, Clarkson pw, Mark Winstanley, et al. Ewing Sarcoma with Novel Translocation t(2;16) Producing an In-Frame Fusion of FUS and FEV. The Journal of molecular diagnostics : JMD. 2007; 9: 459-463.

4. Cantile M, Marra L, Franco R, Ascierto P, Liguori G, Chiara AD, et al. Molecular detection and targeting of EWSR1 fusion transcripts in soft tissue tumors. Medical Oncology (Northwood, London, England). 2013; 30: 412.

5. Shing DC, McMullan DJ, Roberts P, Smith K, Chin SF, Nicholson J, et al. FUS/ERG gene fusions in Ewing's tumors. Cancer Res. 2003; 63: 4568–4576.

6. Szuhai K, Ijszenga M, de Jong D, Karseladze A, Tanke HJ, Hogendoorn PC. The NFATc2 gene is involved in a novel cloned translocation in a Ewing sarcoma variant that couples its function in immunology to oncology. Clin Cancer Res. 2009; 15: 2259-2268.

7. Antonescu CR, Zhang L, Chang N, Pawel BR, Travis W, Katabi N, et al. EWSR1-POU5F1 Fusion in Soft Tissue Myoepithelial Tumors. A Molecular Analysis of 66 Cases, Including Soft Tissue, Bone and Visceral Lesions, Showing Common Involvement of the EWSR1 gene. Genes, chromosomes & cancer. 2010; 49: 1114-1124.

8. Möller E, Stenman G, Mandahl N, Hamberg H, Molne L, van den Oord JJ, et al. "POU5F1, Encoding a Key Regulator of Stem Cell Pluripotency, Is Fused to EWSR1 in Hidradenoma of the Skin and Mucoepidermoid Carcinoma of the Salivary Glands." The Journal of Pathology. 2008; 78–86.

9. Antonescu CR, Nora Katabi, Zhang L, Sung YS, Seethala RR, Jordan RC, et al. "EWSR1-ATF1 Fusion Is a Novel and Consistent Finding in Hyalinizing Clear-Cell Carcinoma of Salivary Gland." Genes, Chromosomes & Cancer. 2011; 559–570.

10. Bridge RS, Rajaram V, Dehner LP, Pfeifer JD, and Perry A. "Molecular Diagnosis of Ewing Sarcoma/primitive Neuroectodermal Tumor in Routinely Processed Tissue: A Comparison of Two FISH Strategies and RT-PCR in Malignant Round Cell Tumors." Modern Pathology: An Official Journal of the United States and Canadian Academy of Pathology. 2006: 1–8.

11. Mhawech-Fauceglia P, Herrmann F, Penetrante R, Beck A, Sait S, Block AM, et al. "Diagnostic Utility of FLI-1 Monoclonal Antibody and Dual-Colour, Break-Apart Probe Fluorescence in Situ (FISH) Analysis in Ewing's Sarcoma/primitive Neuroectodermal Tumour (EWS/PNET). A Comparative Study with CD99 and FLI-1 Polyclonal Antibodies." Histopathology. 2006; 569–575.

Citation: Sadri N, Puthiyaveettil R and Zhang PJ. Study of *EWSR1* Dual Colored Probe FISH Assay on Tumors and Tissues not Known to Have *EWSR1* Translocation on Paraffin Sections: Potential Pitfalls in Interpretation of FISH Signal Separation. Sarcoma Res Int. 2016; 3(3): 1034.