Experimental and Molecular Pathology 90 (2011) 194–200

Contents lists available at ScienceDirect

Experimental and Molecular Pathology

journal homepage: www.elsevier.com/locate/yexmp

Discovery of two novel *EWSR1/ATF1* transcripts in four chimerical transcripts-expressing clear cell sarcoma and their quantitative evaluation

Arturas Jakubauskas [a,*], Vilma Valceckiene [a], Kristina Andrekute [b], Dmitrij Seinin [d], Arvydas Kanopka [e], Laimonas Griskevicius [c,f]

[a] *Department of Molecular and Regenerative Medicine, Hematology, Oncology and Transfusion Medicine Center, Vilnius University Hospital Santariskiu Clinics, Santariskiu 2, Vilnius LT-08661, Lithuania*
[b] *Department of Oncology and Chemotherapy, Hematology, Oncology and Transfusion Medicine Center, Vilnius University Hospital Santariskiu Clinics, Santariskiu 2, Vilnius LT-08661, Lithuania*
[c] *Department of Hematology, Hematology, Oncology and Transfusion Medicine Center, Vilnius University Hospital Santariskiu Clinics, Santariskiu 2, Vilnius LT-08661, Lithuania*
[d] *National Center of Pathology, P. Baublio 5, Vilnius LT-08406, Lithuania*
[e] *Laboratory of Immunology and Cell Biology, Institute of Biotechnology, Vilnius University, V.A. Graiciuno 8, Vilnius LT-02441, Lithuania*
[f] *Clinics of Internal, Family Medicine and Oncology, Faculty of Medicine, Vilnius University, M.K. Ciurlionio 21, Vilnius LT-03101, Lithuania*

ARTICLE INFO

Article history:
Received 7 May 2010
and in revised form 10 December 2010
Available online 24 December 2010

Keywords:
Clear cell sarcoma
Chimerical transcript
EWSR1/ATF1
FISH
qPCR

ABSTRACT

The most common recurrent translocation in clear cell sarcoma *t(12;22)(q13;q12)* results in an *EWSR1/ATF1* chimeric gene. We present a molecular analysis of tumor overgrowing right proximal tibia with bone destruction metastatic to two groin lymph nodes. Fluorescent *in situ* hybridization analysis performed on paraffin-embedded tissue sections of primary tumor sample indicated one rearranged locus of *EWSR1* gene and one additional red signal. Reverse transcription-polymerase chain reaction analysis revealed the presence of four different *EWSR1/ATF1* chimerical transcripts in the tumor sample as well as in both metastatic lymph nodes. Two previously described transcripts *EWSR1exon7/ATF1exon5* and *EWSR1exon8/ATF1exon4*, and two novel transcripts *EWSR1exon7/ATF1exon4* and *EWSR1exon9/ATF1exon4* were identified. Both novel transcripts were out-of-frame fusions and, therefore, most likely had limited biological impact in oncogenesis of clear cell sarcoma. Quantitative evaluation demonstrated unequal distribution of these transcripts, with *EWSR1exon8/ATF1exon4* type being overexpressed.

Introduction

Sarcomas represent a diverse group of mesenchymal tumors with unique clinical features and genetic aberrations. From the point of molecular genetics these genetic aberrations can be divided into those with defined genetic alterations and those with variable genetic alterations. Defined genetic alterations are further separated to those with an oncogenic mutation in defined genes and those with reciprocal chromosomal translocations resulting in oncogenic fusion transcripts (Toguchida and Nakayama, 2009). Sarcomas harboring fusion genes can be classified by the structural–functional features of each fusion gene. Nearly one half of sarcomas are associated with the fusion genes that code for chimerical proteins containing a portion of two representatives of TET protein family: RNA-binding protein EWS (encoded by *EWSR1* gene) and RNA-binding protein FUS (encoded by *FUS* gene) (Tan and Manley, 2009). The chromosome 22q12 breakpoint results in a fusion of *EWSR1* gene with many different partner genes in numerous sarcomas: Ewing's sarcoma family tumors (Khoury, 2008; Szuhai et al., 2009; Wang et al., 2007), desmoplastic small round cell tumor (Liu et al., 2000), extraskeletal myxoid chondrosarcoma (Labelle et al., 1999; Laflamme et al., 2003), myxoid liposarcoma (Bode-Lesniewska et al., 2007; Hosaka et al., 2002; Matsui et al., 2006), angiomatoid fibrous histiocytoma (Rossi et al., 2007) or clear cell sarcoma (CCS).

First described in 1965, CCS is perhaps one of the rarest neoplasms among soft tissue sarcomas (Enzinger, 1965). In case of disseminated disease, complete surgical resection with wide margins with neoadjuvant or adjuvant radiotherapy and chemotherapy remains the optimal treatment (Ferrari et al., 2002; Kuiper et al., 2003). Role of sentinel lymph node biopsy and lymph node resection is still controversial; however, there are successful reports of such treatment strategy (Picciotto et al., 2005). Recent publication from University of Texas M. D. Anderson Cancer Center (MDACC) and the Surveillance, Epidemiology and End Results (SEER) registry compared 5 year disease-specific survival between patients with lymph node metastases and patients with distant metastases and demonstrated significant differences between these two groups of patients (74% (MDACC) and 52% (SEER) versus 14% (MDACC) and 0% (SEER), respectively) (Blazer et al., 2009).

* Corresponding author. Fax: +370 5 2365088.
E-mail address: arturas.jakubauskas@santa.lt (A. Jakubauskas).

0014-4800/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.yexmp.2010.12.005

Both CCS and its mimic malignant melanoma demonstrate significant morphologic overlap as well as similar immunohistochemical features: the presence of melanin, pre-melanosomes and S-100 protein (Dim et al., 2007). Chromosomal translocations *t(12;22)(q13;q12)* and *t(2;22)(q34;q12)* help to differentiate CCS from unusual histological variants of melanoma (Langezaal et al., 2001). *Inter alia*, these translocations are also genetic characteristics of soft tissue tumor of low malignant potential known as angiomatoid fibrous histiocytoma, but there are no overlaps in histological or clinical features (Antonescu et al., 2007; Rossi et al., 2007).

The two partner proteins, which were detected to be fused with *EWSR1* encoded protein in the case of CCS, were transcription factors: cyclic AMP-dependent transcription factor ATF-1 (ATF1) detected in the majority of cases and less frequent cyclic AMP-responsive element-binding protein 1 (CREB1), both belonging to the basic leucine zipper (bZIP) protein family (Hurst, 1995). The fusion protein contains an N-terminal transactivation domain from the RNA-binding protein EWS, but the RNA binding domain in its C-terminal region is replaced with the DNA-binding domain from the corresponding fusion partner protein. To date, four types of *EWSR1*/*ATF1* fusion and a single *EWSR1*/*CREB1* fusion (*EWSR1ex7 (exon 7)* with *CREB1ex7*) have been described (Wang et al., 2009). The most frequently identified *EWSR1*/*ATF1* chimerical transcript designated as type 1 contains an in-frame fusion *EWSR1ex8*/*ATF1ex4*. The in-frame fusions *EWSR1ex7*/*ATF1ex5* and *EWSR1ex8*/*ATF1ex4* were described as type 2 and type 3, respectively. The type 4 corresponds to an out-of-frame *EWSR1ex7*/*ATF1ex7* fusion that was identified only once together with type 1 and type 2 transcripts (Panagopoulos et al., 2002).

Methods

Tumor samples and immunohistochemistry

Formalin-fixed and paraffin-embedded (FFPE) samples of the primary tumor and four groin lymph nodes were presented for morphological and immunohistochemical analysis. Aside from the sections that were stained with hematoxylin and eosin, additional sections were stained with a number of antibodies (Table 1).

FISH analysis

Interphase fluorescent *in situ* hybridization (FISH) analysis of the *EWSR1* rearrangement of the primary tumor was performed using the ON EWSR1 (22q12) Break dual-color probe (containing RH 120772 and D22S1023 loci) and Tissue digestion Kit II (Kreatech Diagnostics, Amsterdam, the Netherlands). FFPE tissue sections of 4 μm thick were mounted on positively charged slides, air dried, and baked overnight at 56 °C. Further processing of slides was performed according to the vendor's recommendations. Slides were analyzed using dual color (TRITS/FITS) filter on Nicon ELIPSE 80*i* fluorescence microscope (Nicon Corporation, Tokyo, Japan) and CytoVisio 3.9 software (Applied Imaging, San Jose, CA, USA). A minimum of 200 cells were scored for the presence of rearranged signals. A split signal pattern was considered positive for the *EWSR1* rearrangement if the distance between the green and the red signals were greater than the diameter of any one signal.

Table 1
Antibodies used in immunohistochemical staining.

Antigen	Antibody/ clone	Dilution	Vendor
S-100	Polyclonal	1:6000	Dako, Glostrup, Denmark
HMB-45	HMB45	1:100	Dako
Melan A	A103	1:70	Dako
Ki-67	MIB-1	1:200	Dako
AsmAct	1A4	1:200	Dako
Desmin	D33	1:250	Dako
Cytokeratin 8/18	5D3	1:100	Novocastra, Newcastle upon Tyne, UK

AsmAct: Anti-Human Smooth Muscle Actin.

Table 2
Primers used for RT-PCR and qPCR assays.

Name	Sequence (5′→3′)	Direction	Position (accession number)
EWS-7F	CCTACAGCCAAGCTCCAAGT	Forward	1046–1065 (NM_005243)
EWS-8F	GGATTTGATCGTGGAGGCAT	Forward	1225–1244 (NM_005243)
EWS-9F	GGTGGCTTCAATAAGCCTGG	Forward	1300–1319 (NM_005243)
ATF-4R	CCTTTTCTGCCCCGTGTATC	Reverse	501–520 (NM_005171)
ATF-5R	GTACTCCATCTGTGCCTGGA	Reverse	644–663 (NM_005171)
ATF-7R	CAACTCGGTTTTCCAGGCAT	Reverse	995–1014 (NM_005171)
CREB-7R	TAGCCAGCTGTATTGCTCCT	Reverse	815–834 (NM_134442)
CCS-7/4F	CAGCTACGGGCAGCAGAAAAA	Forward	*EWSR1ex7/ATF1ex4* junction site
CCS-7/5F	CAGCAGATTGCCATTGCCCC	Forward	*EWSR1ex7/ATF1ex5* junction site
CCS-8/4F	GACGCGGTGGAATGGGAAAAA	Forward	*EWSR1ex8/ATF1ex4* junction site
qATF-4R	GCAATGTACTGTCCGCTGCT	Reverse	591–610 (NM_005171)
qATF-5R	ATCAGAGGTCTGTGCATACTGA	Reverse	725–746 (NM_005171)
UBC-F	TGGGTCGCAGTTCTTGTTTG	Forward	403–422 (NM_021009)
UBC-R	CCTTCCTTATCTTGGATCTTTGCC	Reverse	529–552 (NM_021009)

Primers

Primers used for reverse transcription-polymerase chain reactions (RT-PCRs) and quantitative real-time PCRs (qPCRs) are presented in Table 2. Primer pairs were designed using Primer3Plus software (Untergasser et al., 2007). Primer sequences were based on the *EWSR1* (accession number NM_005243), *ATF1* (NM_005171), *CREB1* (NM_134442), and *UBC* (coding for ubiquitin; NM_021009) mRNA sequences. All oligonucleotides were synthesized by Metabion (Martinsried, Germany).

RT-PCR analysis

Total RNA was extracted from FFPE sections containing tumor tissue. The extraction was performed using PureLink FFPE Total RNA Isolation Kit as directed by the vendor (Invitrogen, Carlsbad, CA, USA). RNA (1 μg) was reverse transcribed in 20 μl reaction volume using random hexamers and First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania) according to the vendor's recommendations and diluted up to 100 μl with deionized water after reaction.

PCRs were performed in a 20 μl reaction volume containing Maxima Probe qPCR Master mix (Fermentas), 0.2 μM final concentration of each primer, and 5 μl cDNA solution. Experiments were done in duplicates. Uracil-DNA glycosylase (Fermentas) was added into each reaction mix (0.4 units) to prevent PCR cross-contamination. All PCRs were run on a TProfessional Standard thermocycler (Biometra, Goettingen, Germany) using the same cycling profile: uracil-DNA glycosylase treatment at 50 °C for 2 min; initial denaturation step at 94 °C for 5 min; 40 cycles of denaturation at 94 °C for 15 s, annealing at 60 °C for 15 s, extension at 72 °C for 15 s; the final extension step was 72 °C for 10 min. Each primer pair was tested in a reaction setup without cDNA sample (negative template control).

RT-PCR products were visualized by QIAxcel System using DNA Screening Gel Cartridge (Qiagen, Irvine, CA, USA). NoLimits 50 bp and 1000 bp DNA fragments (Fermentas) served as the first and the last reference markers, respectively. GeneRuler 50 bp DNA Ladder (Fermentas) was used for the sizing of DNA fragments.

Sequence analysis

The resultant RT-PCR fragments were purified from agarose gel using GeneJET Gel Extraction Kit (Fermentas), and cloned into