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Variability in Gene Expression Patterns of Ewing Tumor Cell Lines Differing in EWS-FLI1 Fusion Type

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SUMMARY: Type 1 and type 2 EWS-FLI1 fusion products result from variation in breakpoint locations arising from the t(11;22)(q24;q12) recurrent chromosomal translocation in Ewing's sarcoma family tumors (EFT). Previously, studies from our institution (updated in the present communication at a median follow-up of more than 6 years) and others suggested a prognostic difference for EFT patients with localized disease depending on the type of EWS-FLI1 fusion present in the tumor. It has been suggested that the observed clinical discrepancies result from different transactivation potentials of the various EWS-FLI1 fusion proteins. In an attempt to identify genes whose expression levels are differentially modulated by structurally different EWS-FLI1 transcription factors, we have used two related PCR-based subtractive approaches, cDNA representational difference analysis (cDNA-RDA) and linker-capture subtraction (LCS) to compare transcript representations in cDNA pools of type 1 versus type 2 EFT cell lines. About 800 clones obtained by the two approaches were analyzed by dot blot hybridization to cDNA pools. Eighty-six clones showing the highest variability in signal intensities on the dot blots were further hybridized to individual EFT cell line RNAs on Northern blots, and four of them were additionally studied by real-time quantitative PCR (RTQ-PCR). Although interindividual variations in gene expression patterns in the range of one- to several-fold were observed, no correlation to specific EWS-FLI1 fusion types could be identified. Among the genes differentially expressed in individual EFT cell lines are several previously implicated in tumor growth, invasion, and metastasis. Although our data may have revealed candidate genes whose composite expression pattern may be relevant for the biology of individual EFT, they do not support a role of distinct EWS-FLI1 fusion types for EFT prognosis based on different transactivation potentials. (*Lab Invest 2000, 80:1833–1844*).

The course of normal cellular development, as well as that of neoplastic transformation leading to cancer, is believed to be driven by changes in gene expression. The identification and characterization of differentially expressed genes associated with different prognostic subtypes of cancer may help us to better adjust treatment to the specific biology of the disease in individual patients and discover new targets for therapeutic intervention. Methods to identify variably expressed genes in tissues have included differential screening of cDNA libraries, various forms of subtractive hybridization, and differential display reverse transcriptase polymerase chain reaction (DDRT-PCR) (Liang and Pardee, 1992). DDRT-PCR relies on random-primed amplification of total RNA populations aiming at the identification and isolation of differential bands in sequencing gels. Although it is a powerful method, it is hampered somewhat by a relatively large number of false positives and its preferential isolation of 3′ untranslated regions. Subtractive hybridization using conventional methods has been a very tedious approach, and this has led to a series of developments in which PCR has been included (Byrne et al, 1995; Hubank and Schatz, 1994; Wang and Brown, 1991; Zeng et al, 1994). Representational difference analysis (RDA) is a combination of subtraction and kinetic enrichment, coupled with subsequent amplification, which was originally developed to isolate differences between complex DNA genomes (Lisitsyn and Wigler, 1993; Lisitsyn et al, 1995). Whereas DDRT-PCR amplifies fragments from all represented mRNA species, RDA has the advantage of eliminating fragments common to both populations, leaving only the differences.

The Ewing's sarcoma family tumors (EFT) are characterized on the molecular level by the t(11;22) or t(21;22) recurrent chromosomal translocation in about 85% and 10% of cases, respectively. In rare EFT other translocations also involving chromosome 22 have been observed. These chromosomal rearrangements result in the fusion of the DNA-binding domain of a member of the *ets* transcription factor family to the amino terminal domain of a putative RNA-binding protein, EWS, of as yet unknown function. The resultant chimeric protein is a potent transcriptional activator. The two most common fusions join *EWS* exon 7 in frame with either exon 6 (type 1 fusion) or exon 5 (type 2 fusion) of FLI1. EWS-FLI1 transforms NIH 3T3 cells,

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which suggests that this molecule plays an active role in tumorigenesis (Lessnick et al, 1995; May et al, 1993).

Ewing tumor patients can be assigned to one of two different risk groups depending on the extent of the disease at diagnosis. Although about 60% of patients with localized EFT can be cured by modern multimodal treatment regimens, patients with metastases are at an 80% risk of succumbing to the disease (Kovar, 1998). A significantly better treatment outcome was reported for a limited number of patients with localized disease when the tumors expressed a type 1 EWS-FLI1 fusion compared with tumors that expressed a non-type 1 EWS-FLI1 fusion. This introduced the question of whether *EWS-FLI1* gene fusion type might serve as a prognostic molecular indicator in this group of patients (Zoubek et al, 1996). More recently, data on independent patient cohorts were obtained that support the idea of a better treatment outcome (de Alava et al, 1998) at the same time that they contradict a putative prognostic impact of EWS-FLI1 fusion type (Delattre, personal communication).

Recently, a difference in the transactivation potential of different EWS-FLI1 fusion types has been described. Reporter gene assays performed in EWS-FLI1-transfected Hela and NIH 3T3 cells, as well as in EFT cell lines of different *EWS-FLI1* fusion type, revealed an approximately two-fold weaker transcriptional activity of type 1 versus non-type 1 EWS-FLI1 on an *ets* responsive promoter element. This suggests that if different *EWS-FLI1* fusion types are associated with different clinical behavior, this association may be caused by qualitatively or quantitatively different EWS-FLI1-dependent gene expression patterns (Lin et al, 1999). Although some advances have been made in isolating EWS-FLI1 target genes (Braun et al, 1995; Hahm et al, 1999; May et al, 1997; Thompson et al, 1996), no data exist regarding differential expression of genes in EFT carrying the different fusion products. To define the biological basis for the observed differences in clinical behavior, we sought to define discrepancies in gene expression profiles between EFT cell lines of the most frequent fusion types 1 and 2 using two related PCR-based subtraction strategies: cDNA representational difference analysis (cDNA-RDA) and linker-capture subtraction (LCS). We now report the identification of several genes whose composite expression differences between EFT cell lines carrying the two different EWS-FLI1 fusion types may reflect differences relevant to the course of the disease in patients. However, so far, no fusion-type associated differences in gene expression patterns that might account for the reported clinical heterogeneities were identified.

Results

Identification of Genes Differentially Expressed between Type 1 and Type 2 EFT Cell Lines

An earlier clinical study compared type 1 EWS-FLI1–expressing EFT to a heterogenous group of tumors expressing a variety of other fusion types at a median observation time of 30 months (Zoubek et al, 1996). Figure 1A presents an update of treatment results for the cohort of patients with localized disease described previously. After a median observation time of more than 6 years, Kaplan-Meier curves for the two groups still diverged from each other, with a probability for relapse-free survival at 5 years of 0.64 and 0.41, respectively ($p = 0.051$). However, the predominant gene rearrangements in the non-type 1 group of patients lead to type 2 *EWS-FLI1* gene fusions (67%). Figure 1B depicts a comparison between type 1 and type 2 EWS-FLI1–expressing localized tumors only. Kaplan-Meier curves for these more tightly defined groups of patients reflected the results obtained for the whole group of patients with localized disease, although the difference in relapse-free survival (probability at 5 years: 0.64 for type 1 *EWS-FLI1* fusions versus 0.40 for type 2 fusions) lacked statistical significance ($p = 0.156$), possibly because of the relatively small number of patients. For simplicity, we therefore decided to restrict our comparison of gene expression patterns to type 1 and type 2 EWS-FLI1–expressing EFT.

To identify genes differentially expressed in association with distinct EWS-FLI1 fusion types, the starting material for the subtractive screen had to meet three criteria: (a) a high tumor-cell content to avoid the masking of tumor-specific gene expression by con-

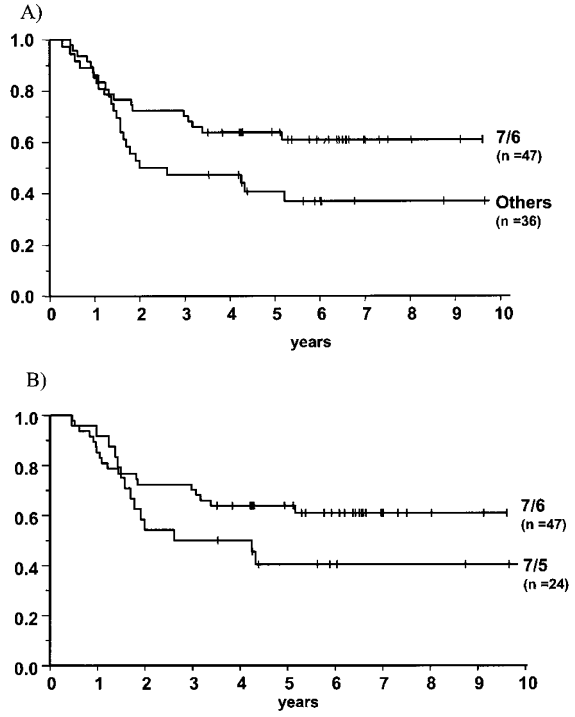

Figure 1.

Kaplan-Meier curves for (*A*) update of relapse-free survival (RFS) of EFT patients with localized disease of either type 1 (7/6) ($p^* = 0.64 \pm 0.07$) or other ($p^* = 0.41 \pm 0.08$) EWS-FLI1 exon combinations at a medium observation time of 6 years and 4 months, $p = 0.051$, and (*B*) RFS of patients with either type 1 ($p^* = 0.64 \pm 0.07$) or type 2 (7/5) ($p^* = 0.40 \pm 0.10$) EWS-FLI1 exonic fusions at a median observation time of 6 years and 2 months, $p = 0.156$. p^*, Probability of survival at 5 years.

taminating healthy tissue, (b) availability in sufficiently high amounts to allow for the confirmation of differential expression by complementary approaches (ie, repeated Northern blotting and real-time PCR), and (c) minimization of random interindividual differences in gene expression. We therefore chose to use cell lines instead of primary EFT. A mixture of equal amounts of total RNA from each of seven exponentially growing cell lines per individual fusion type has been employed to enrich for fusion-type–associated differences in gene expression relative to intratype variations. Two closely related PCR-coupled subtractive hybridization procedures were conducted, cDNA-RDA and LCS. Subtraction was performed in both directions, type 1 minus type 2 cell lines and vice versa. Initially, the subtraction process in each approach was carried out three times, using the tester:driver amplicon ratios of 1:100, 1:800, and 1:8,000 in three successive rounds of subtraction, respectively. Because the PCR products from the third round of subtraction did not show any enhancement in intensity over the second round products, as was previously observed (Chang et al, 1998; Geng et al, 1998; O'Neill and Sinclair, 1997), PCR fragments from the second round of subtractive hybridization were cloned into pGEM-T Easy (Promega, Madison, Wisconsin) for further analysis to achieve a high degree of sensitivity and diversity (Welford et al, 1998).

Dot-Blot Hybridization and Northern Blot Analysis

Eight hundred white colonies for each subtracted library were picked at random and were analyzed by PCR to confirm the presence and the respective sizes of inserts. Eight clones failed to produce PCR products, and five clones did not have any inserts. With the remaining clones, we prepared replica DNA dot-blots and probed them with the radiolabeled type 1 and type 2 driver cDNA pools, respectively (Fig. 2). A visual comparison of the hybridization intensity for each clone in the two replica membranes allowed for a rough estimate of relative transcript abundancies in type 1 versus type 2 cDNA pools and was used to isolate candidate positives described in Tables 1 and 2. Most of the genes that segregated with the different fusion types are known genes, some of which have been reported to be differentially expressed in cancers and to affect cell proliferation and tumor progression (ie, elongation factor 1-alpha, translational controlled tumor protein, v-fos transformation effector protein). Twelve clones, 11 of which were picked by LCS and 1 by cDNA-RDA, had no matches to any sequence in the Genbank. When used as probes in Northern blot analyses, no signals were obtained. Several high-abundance transcripts, such as those from cytoskeletal-associated genes and from some ribosomal and housekeeping genes, were also picked up by the two differential screening methods, but many of them were found to be constitutively expressed in cells irrespective of the fusion type.

Northern blot analyses of RNAs from individual type 1- and type 2-expressing cell lines (originally contained in the tester and driver cDNA pools) using selected clones as probes was performed to confirm differential expression of candidate positives from the dot-blot assays (Fig. 3). None of the 86 different clones yielded a consistent EWS-FLI1 fusion type-specific difference in expression levels. Rather, very heterogenous expression levels with intratype and intertype variations were found. The expression of the Fibronectin gene, which was obtained in the type 1 minus type 2 subtraction, was found to be more than 100-fold higher in the type 1 cell line A673 and not expressed in any of the type 2 cell lines. Steady-state transcript levels of individual genes were quantified by phosphor-imaging and normalized by comparison to the hybridization signal obtained with an 18S rDNA probe. The degree of expression differences of 13 confirmed positives was found to range from one- to about two-fold (Fig. 4). Statistical analysis, using the Kruskal-Wallis test, of results obtained by phosphorimaging did not give any significant differences in the expression levels of type 1 versus type 2 cell lines for any isolated clone, indicating lack of correlation with EWS-FLI1 fusion type. For most clones, distinctively high transcript abundance in single cell lines may explain their overrepresentation in starting RNA pools, which enables their isolation by high-sensitivity subtractive screening.

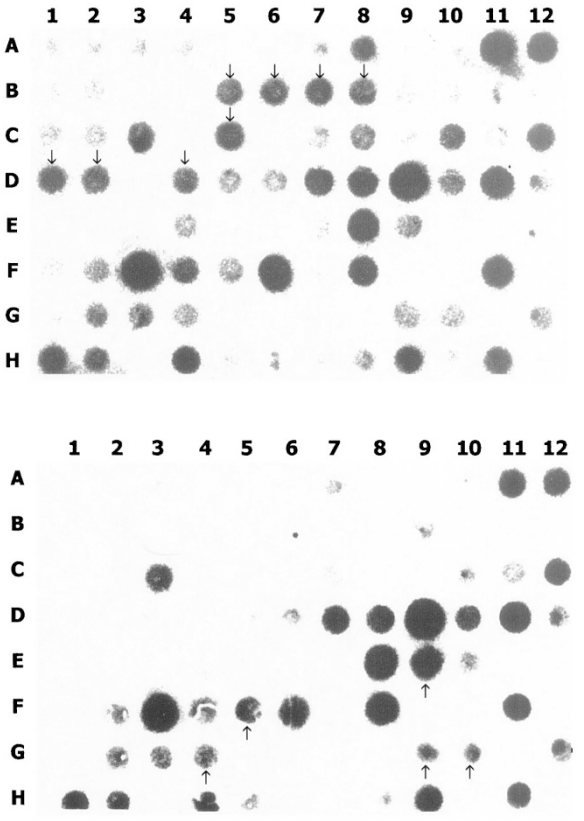

Figure 2.
Dot-blot screening assay for differentially expressed genes. Replica DNA dot-blots were prepared from randomly picked colonies for type1 minus type2 (A1-D12) and type 2 minus type1 (E1-H12) subtractions and probed with labeled driver cDNAs for type 1 (upper panel) and type 2 cells (lower panel). Clones differentially represented are indicated by *arrows*.

Table 1. Ewing Tumor Type 1 — Type 2 Clones

Clone #	Blast Identity	Accession #	Redundancy[a]
1*	Hu. clone 24651 mRNA	gb/AF070648	5
2*	Hu. L23 mRNA for putative Ribosomal RNA	emb/X53777	3
3*	Hu. PAC clone DG0899B21 from 7p15-p21	gb/AC004008	1
4*	Hu. BAC clone RG180F08 from 7q31	gb/AC002431	1
5*	Hu. type-IVA cAMP-specific phosphodiesterase	gb/S75213	1
6*	Hu. KIAA0026 gene	dbj/D14812	9
7*	Hu. seq. from PAC 211D12 on 20q12-13.2	emb/Z93016	1
8*	Hu. Nucleosome assembly protein NAP	gb/M86667	2
9*	C. elegans cosmid E02H1	emb/Z47075	1
10*	Hu. Chromosome 16 BAC clone CIT987SK-A-101F10	gb/AC002550	1
11*	Hu. Xq28 cosmid U247A3	gb/U73465	1
12*	C. elegans cosmid WO9C3	gb/U88178	1
13*	Hu. mRNA for Nucleolar Protein hNop56	emb/Y12065	1
14*	Hu. V-fos transformation effector protein (Fte-1)	gb/M84711	1
15*	C. elegans cosmid F41C6	gb/U39745	1
16	Cloning vector pGEM-5Zf(+)	embX65308	1
17	Hu. ribosomal protein L5 mRNA	gbU14966	1
18	Hu. ckshs1 mRNA for Cks1 protein homologue	embX54941	1
19	Hu. skin collagenase mRNA	gbM13509	2
20	Hu. alpha-tubulin isoform 1 mRNA	gbAF081484	1
21	Hu. mitochondrial DNA	embX93334	1
22	Hu. DNA seq. from clone 756G23 on chrom. 22q13.31	emAL035681	1
23	Hu. mRNA for 90-kDa heat shock protein	embX15183	1
24	Hu. Ubiquitin-activating enzyme E1 (UBE1) mRNA	gbM58028	1
25	Hu. mRNA for fibronectin	embX02761	1
26	Hu. mRNA for transl. controlled tumor protein (TPT1)	embX16064	1
27	Hu. clathrin, heavy polypeptide-like 2 (CLTCL2) mRNA	dbjD21260	1
28*	Hu. Elongation factor 1-alpha	emb/X03558	1
29*	C. elegans cosmid C31H5	emb/Z93778	1
30*	Hu. clone UWGC:y2c074 from 6p21	gb/AC004203	1
31*	Hu. Ribosomal Protein S4 isoform (RPSX4) mRNA	gb/M58458	1
32*	Hu. Ribosomal Protein S24	gb/U12202	1

[a] Redundancy refers to the number of individual cDNA clones matching the same gene.

* Clones identified by linker-capture subtraction (LCS); clones without asterisks identified by representational difference analysis (RDA); Hu, human.

Validation of Northern Blot Data with Real-Time Quantitative PCR

To further investigate the reliability of our Northern blot data, we measured the expression levels of four candidate clones whose relative expression levels between cell lines with the different fusion transcripts were close to significance, using the TaqMan 5′nuclease fluorogenic quantitative PCR assay (RTQ-PCR). Figure 5 illustrates the expression pattern and the quantitative expression level of each of the four genes as determined by RTQ-PCR. Statistically, no significant differences in the steady state transcript levels could be detected for all the genes analyzed. Although the transcriptional level of a given gene varies dramatically, no group-specific up- or down-regulation could be detected.

Discussion

In a series of 83 localized EFT, treated according to the European Intergroup Cooperative Ewing's Sarcoma Study (CESS and EICESS) protocols, we observed an *EWS-FLI1* fusion type-associated difference in long-term event-free survival. This difference was also reflected by a comparison between type 1- and type 2-expressing localized tumors only, although statistically the difference was not significant and was restricted to localized disease. Primary metastatic disease was generally associated with an adverse prognosis (data not shown). This study was performed to evaluate the molecular basis for the fusion type-associated difference in the clinical course of the disease. In a previously published study by Lin et al (1999), reporter gene transactivation by type 1 and other types of *EWS-FLI1* fusions in EFT cell lines was found to differ up to two-fold. To achieve maximum sensitivity for differences in endogenous gene expression of EFT cells, we focused on homogenous tumor cell populations by using EFT cell lines. Although in patients, several factors may influence prognoses differently and EWS-FLI1 fusion type-associated gene expression differences may impact on localized disease only, we expected these differences to be present in EFT cell lines regardless of the extent of the disease in the patients from which the cell lines were derived. Our primary goals were to rapidly screen

Table 2. Ewing Tumor Type 2 − Type 1 Clones

Clone #	Blast Identity	Accession #	Redundancy
1*	Hu. gamma-actin gene	gb/M16247	2
2*	Hu. beta-2 microglobulin gene	emb/V00567	4
3*	Hu. KIAA0026 gene	dbj/D14812	1
4*	Hu. Ribosomal Protein L34 mRNA	gb/L38941	2
5*	Hu. tetrameric Ubiquitin pseudogene	emb/X07499	1
6**	Hu. Ribosomal Protein L9 mRNA	gb/U21138	4
7*	Hu. Mitochondrial DNA	dbj/038112	1
8*	Hu. mRNA for Seryl-tRNA Synthetase	emb/X91257	4
9*	Hu. acidic Ribosomal phosphoprotein PO	gb/M17885	1
10*	Hu. NADH-ubiquinone oxidoreductase 15kDa Subunit	gb/AF047434	1
11*	Hu. scar protein mRNA	gb/M22146	1
12*	Hu. mRNA for ATP synthase	emb/X83218	1
13**	Hu. Elongation factor 1-alpha gene	gb/J04617	7
14*	Hu. alpha-tubulin mRNA	gb/K00558	1
15*	Hu. T-cell cyclophilin gene	emb/Y00052	3
16*	Hu. hnRNP core protein A1	emb/X79536	1
17*	C. elegans cosmid T11F9	emb/Z74042	1
18*	Hu. mRNA for Nucleolar protein hNop56	emb/Y12065	4
19*	Hu. Ribosomal Protein L7	gb/L16558	2
20**	Hu. Translationally controlled tumor protein clone 04	gb/L13806	7
21**	Hu. Mitochondrial cytochrome oxidase subunit II	gb/M25171	3
22	Hu. cytoplasmic dynein light chain 1 (hdlc1) mRNA	gbU32944	1
23	Hu. gene for ribosomal protein L38	embZ26876	1
24	Hu. ubiquitin-activating enzyme E1(UBE1) mRNA	gbM58028	1
25	Hu. alpha enolase mRNA	gbM14328	1
26	Hu. BTK region clone 2f10-rpi mRNA	gbU01925	1
27	Hu. mRNA for homologue to yeast ribos. protein L41	embZ12962	1
28	Hu. mRNA for ribosomal protein S11	embX06617	1
29	Hu. XP2NE ribosomal protein S3 (rpS3) mRNA	gbU14991	1
30	Hu. mRNA for DNA-binding protein, TAXREB107	dbjD17554	2
31	Hu. chromosome 9q34, clone 255A6, complete seq.	gbAC004530	1
32	Hu. G-protein	gbM16514	1
33	Hu. mRNA for beta-actin	embX00351	1
34	Hu. transformer-2-beta (SFRS10) gene	gbAF057159	4
35	Hu. RPS3a mRNA	gbM77234	1
36	Hu. FLI-1 mRNA	embX67001	3
37	Hu. mRNA f. phosphatidylinositol transfer prot.(PI-Tbβ)	dbjD30037	1
38	Hu. RPS16 mRNA	gbM60854	1
39	Hippoglossus hipglossus microsatellite HhiC17	gAF133244.1	1
40	Hu. mRNA for Na, K-ATPase a subunit	embX04297	1
41	Hu alpha-tubulin isoform 1 mRNA	gbAF081484	1
42	Hu. testis enhanced gene transcript (TEGT) mRNA	embX75861	1
43	Hu. mRNA for zinc finger protein 198 (ZNF198)	emAJ224901	1
44	Hu. calpain, small polypeptide (CAPN4) mRNA	Nm_001749.1	1
45	Hu.putative translation initiation factor A121/Sui1	gAF100737.1	1
46	Hu. epithelial membrane protein 1 (EMP1) mRNA	embY07909	1
47	Hu. mRNA for calmodulin	dbjD45887	1
48	Hu. MHC prot. homologue to chicken B complex mRNA	gbM24194	1
49	Hu. lactate dehydrogenase B (LDHB) mRNA	NM_002300.1	1
50	Hu. DNA seq. F. clone 90L6 on chrom.22q11.21-11.23	embZ97353	1
51	Hu. metabotropic glutamate receptor 1 alpha mRNA	gbU31215	1
52	Hu. mRNA for mito. Ubiquinone-binding protein (QP-C)	embX13585	1
53	Hu. HSP-89 alpha-delta N mRNA	gb/AF028832	2
54*	Hu. mRNA for radixin	emb/X60672	1
55*	Hu. Ribosomal Protein S6	gb/J03537	1
56	Hu. NADH-ubiquinone oxidoreductase subunit CI-B14	gbAF047182	1
57	Hu. c-myc transcription factor (puf) mRNA	gbL16785	1
58	Hu. JTV-1mRNA	gbU24169	1
59	Hu. Voltage-dependent anion channel isoform2 (VDAC)	gbL06328	1

* Clones identified by LCS; clones without asterisks identified by RDA; ** clones identified by both RDA and LCS.

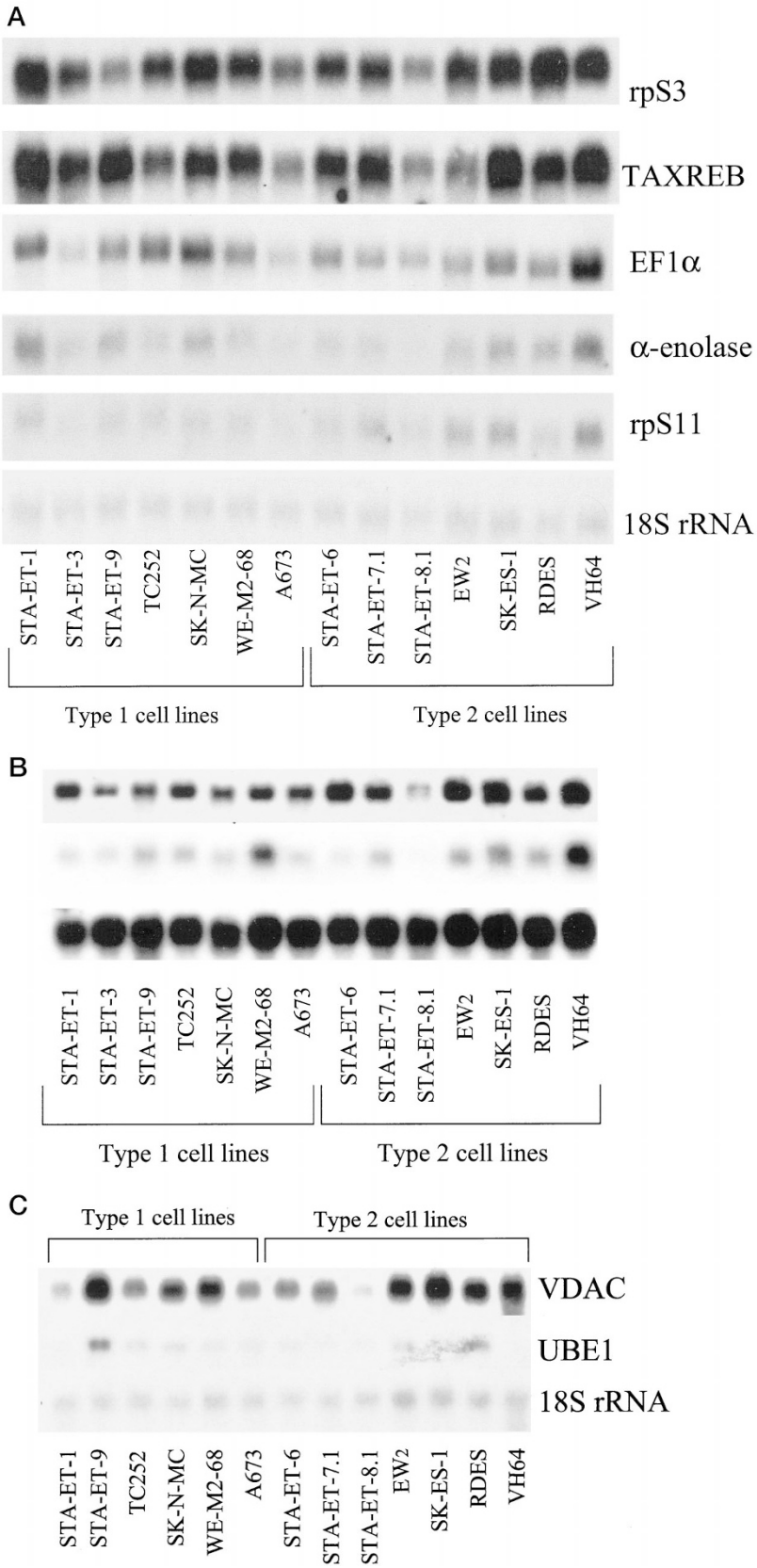

Figure 3.

Northern blots to confirm the differential expression of candidate positive clones detected by RDA and LCS (*) in type 1 vs type 2 EWS-FLI1 expressing Ewing tumor cells. *A* to *C*, Representative fragments recovered from type 1 minus type 2 subtraction (rpS3, TAXREB107, α-enolase, rpS11, UBE1, α-tubulin, rpL9) and from type 2 minus type 1 subtraction (EF1α, VDAC) are shown. Total RNA form either cell type used in the pool was probed with radiolabeled cDNA from candidate positives as shown in autoradiograms. 18S rRNA was used to control for equal sample loading.

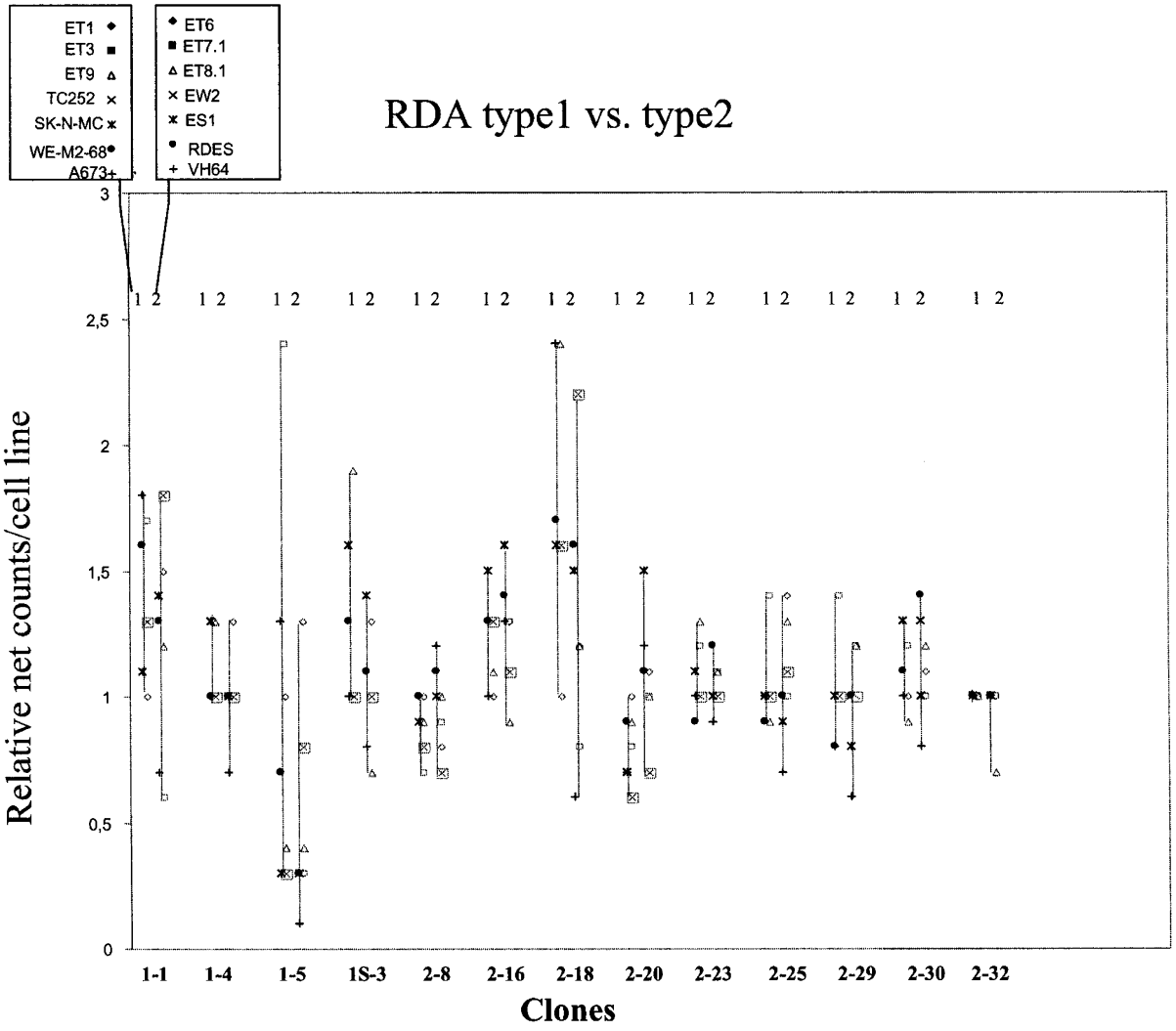

Figure 4.

Graphic representations of data obtained by phospho-imager analysis of chosen Northern blots hybridized with two RDA probes. Data are normalized to 18S rRNA control. Type 1 minus type 2 clones: 1-1 (Human mitochondrial gene), 1-4 (UBE1), 1-5 (Human fibronectin gene), 1S-3 (VDAC). Type 2 minus type 1 clones: 2-8 (rpS3a), 2-16 (rpS16), 2-18 (Human mitochondrial cytochrome oxidase subunit II), 2-20 (TAXREB107), 2-23 (Human TEGT gene), 2-25 (CAPN4), 2-29 (Human calmodulin gene), 2-30 (Human MHC gene), 2-32 (Human clone 90L6 on 22q11.21).

pools of EFT cell lines in a pairwise fashion for differential gene expression and to analyze the expression patterns of selected candidate genes in all EFT cell lines individually before testing EFT primary tissues. We further assumed that *p53* tumor suppressor gene mutations and *INK4A* deletions, which have been much more frequently encountered in EFT cell lines than in primary tumors (Kovar et al, 1997), would not influence our study because they were present in the majority of cell lines included in both pools compared. However, we cannot exclude the possibility that the expression of certain genes regulated by EWS-FLI1 is also affected by alterations in *p53* and *INK4A* tumor suppressor genes, which have been proposed to be associated with bad prognoses in a subset of EFT patients (de Alava et al, 2000; Kovar et al, 1997; Wei et al, 2000).

Some modifications of both the cDNA-RDA and LCS procedures were applied because the selectivity and sensitivity of the two strategies applied is greatly influenced by the ratio of tester to driver cDNA pools and by the number of consecutive subtractions (Welford et al, 1998). Although the validity of the RDA and LCS methods in detecting expression differences with high sensitivity could be confirmed, the majority of the clones derived from our screen showed interindividual variation rather than *EWS-FLI1* fusion type specificity. Expression differences were found to be in the range of up to two-fold, as indicated by phosphor-imaging of hybridization signals on Northern blots and by RTQ-PCR. Based on the method's sensitivity, significant differences in gene expression patterns segregating with specific *EWS-FLI1* fusion type should have been selected. However, if only a small set of genes exist that are differentially affected by the different EWS-FLI1 fusion proteins, we may have missed the relevant candidates by analyzing only about 800 clones on dot blots. The 112 genes that were used for individual analyses on Northern blots were selected because they displayed the highest discrepancies in signal

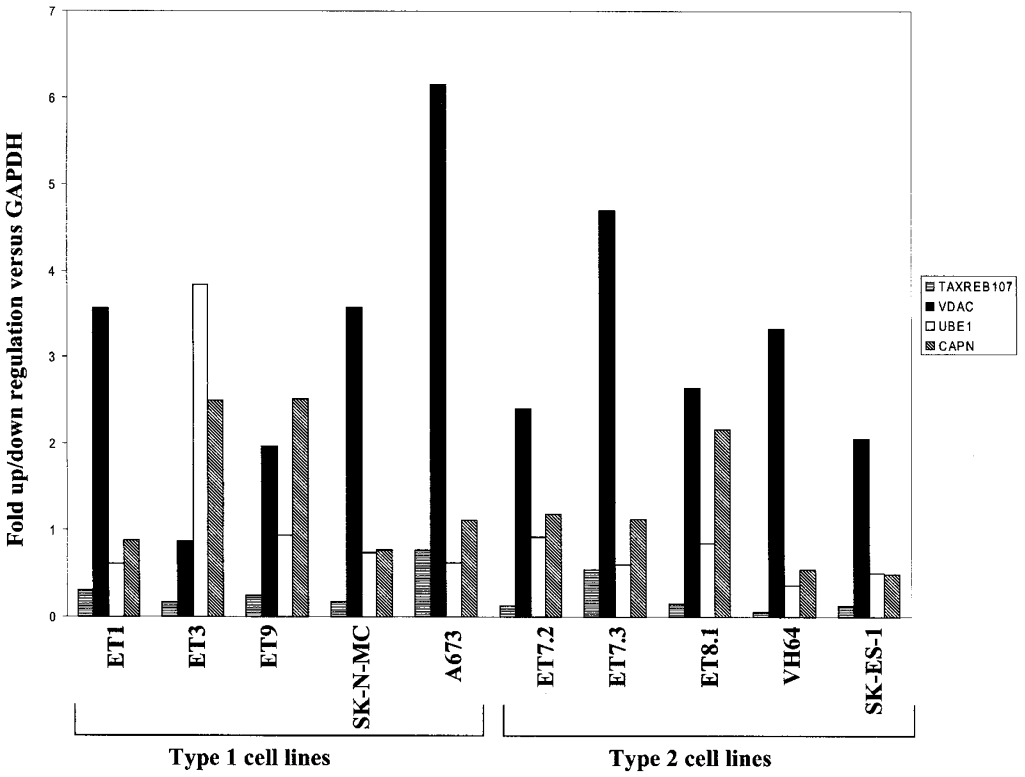

Figure 5.

Real-time quantitative PCR (TaqMan analysis) data obtained using 4 RDA clones (VDAC, TAXREB107, CAPN, and UBE1). Amplification plots of fluorescence intensity of RDA clones relative to GAPDH in EWS-Fli1 type 1 and type 2 cell lines.

intensities on dot blots. In fact, when tested on individual cell line RNAs, even these clones showed only two-fold variations in gene expression. Their overrepresentation in one, as opposed to the other, cDNA pool was most likely caused by increased expression levels in single cell lines, as has been observed for the Fibronectin gene in the A673 type 1 cell line. Thus, among the 800 clones analyzed, it is unlikely that we have missed clones that are significantly more highly expressed in cell lines of one as compared to those of the other EWS-FLI1 fusion type with consistency.

The results presented here serve to illustrate the wide array of genes expressed to different degrees in EFT. Interestingly, genes identified thus far are from several functional categories, including cytoskeletal components, chromatin remodeling molecules, nuclear proteins (transcription factors and DNA processing enzymes), ion channels, protein processing, protein transport and folding molecules, extracellular proteins, signaling molecules, and growth and differentiation regulators.

The elucidation of the role of each of these candidate genes in the EFT cell phenotype is beyond the scope of this series of experiments. Several genes identified in our study have been reported to be differentially expressed in cancers and to affect cell proliferation and tumor progression. For example, the expression of elongation factor 1-alpha (Edmonds et al, 1996), translationally controlled tumor protein (Baudet et al, 1998), and the v-fos transformation effector protein (Lecomte et al, 1997) have been reported to be altered in several tumors. It has been recently reported that cleavage of p35, the neuron-specific activator of cyclin-dependent kinase 5 (cdk5), to p25 by calpain (one of the isolated clones) may be involved in the pathogenesis of Alzheimer's disease (Lee et al, 2000). It is therefore possible that some of these genes contribute to the biology of individual EFT independently of EWS-FLI1 fusion type. It cannot be established at this stage whether composite expression patterns of these genes may differ in EFT of different prognostic subgroups. The different clones obtained for each fusion type from both cDNA-RDA and LCS could be used on cDNA microarrays for hybridization to a large number of individual EFT to identify such composite expression patterns. It may also be possible that there is no difference in the transcriptional activity of the various EWS-FLI1 chimeric transcription factor types on endogenous gene expression in EFT and that the transactivation potentials of type 1 and type 2 fusion types, so far identified in reporter gene assays only, do not contribute to different courses of disease. Instead, there may be a transactivation independent function for EWS-FLI1, as suggested recently (Jaishankar et al, 1999), that may be influenced by different fusion type structures. Alternatively, it cannot be excluded that variability in EWS-FLI1 architecture does not affect EFT biology at all, because the clinical results previously obtained by Zoubek et al (1996) and others (de Alava et al, 1998) could not be confirmed so far for a cohort of patients treated in France (Olivier Delattre, personal communication). Thus, the impact

of EWS-FLI1 fusion type on prognosis remains disputable and requires prospective evaluation on greater numbers of patients.

Based on the findings presented in this study, it could be deduced that if we had chosen at random a cell line for each fusion type for both the RDA and LCS analyses, the probability of obtaining clones that will clearly show significant differences in their expression level between the two materials would have been very high. However, our results suggest that the majority of these differences would have presumably resulted from intraindividual, rather than from EWS fusion type-associated, variation in gene expression; that is, they would have reflected only the individual situation of the cell line. On this basis, we have abstained from analyzing some of the candidate clones by RTQ-PCR, using only the primary tumor samples at our disposal. Our data, therefore, call for caution in interpreting the results from comparisons of single tumor specimens as revealing general biological differences in tumor behavior.

Materials and Methods

Cell Lines and Fusion Types

EFT cell lines were routinely maintained in RPMI-1640 (GIBCO BRL, Gaithersburg, Maryland) supplemented with 10% fetal bovine serum, penicillin (0.1 mg/ml), and streptomycin (0.1 mg/ml) in 5% CO_2 at 37° C. All cell lines used in this study expressed EWS-FLI1, as determined by reverse transcriptase polymerase chain reaction (RT-PCR) (Zoubek et al, 1996). Cell lines SK-N-MC, a generous gift from Dr. J. Biedler (Memorial Sloan-Kettering Cancer Center, New York), WE-68 and WE-M2-68, kindly provided by Dr. F. van Valen (Department of Pediatrics, University of Münster, Münster, Germany), and TC-252, a gift of Dr. T. Triche (Department of Pathology, Children's Hospital, Los Angeles, California), as well as A673, from the American Type Culture Collection (ATCC, Rockville, Maryland), expressed type 1 EWS-FLI1. Cell lines IARC-EW2, kindly supplied by Dr. G. M. Lenoir (International Agency for Research on Cancer, Lyon, France), VH64, provided by Dr. F. van Valen (Department of Pediatrics, University of Münster), RDES and SK-ES1 (ATCC) carried type 2 EWS-FLI1 fusions. Among the cell lines established in our institute, STA-ET-1, STA-ET-3, and STA-ET-9 expressed type 1, and STA-ET-6, STA-ET-7, and STA-ET-8 expressed type 2 EWS-FLI1 chimeric products.

RNA Isolation and cDNA Synthesis

Poly(A)+ RNA was extracted from the total RNA of a pool of seven different RNAs for each fusion type through oligo$(dT)_{25}$-Dynabeads (Dynal, Hamburg, Germany) according to the manufacturer's recommendations. cDNA was synthesized from 1 μg of poly(A)+ RNA employing the SMART cDNA synthesis technology (Clontech, Palo Alto, California). Briefly, a modified oligo dT primer (cDNA synthesis [CDS] primer, with an *Rsa*I site to facilitate removal of these identical sequences from the PCR-amplified cDNA molecules) primes the first strand cDNA synthesis reaction. When reverse transcriptase (RT) reaches the 5′ end of the mRNA, the enzyme's terminal transferase activity adds a few additional nucleotides, primarily deoxycytidine, to the 3′ end of the cDNA. A SMART™ oligonucleotide, which has an oligo(G) sequence at its 3′ end, base-pairs with the deoxycytidine stretch, creating an extended template. RT then switches templates and continues replicating to the end of the oligonucleotide. The resulting full-length, single-stranded cDNA contains the complete 5′ end of the mRNA, as well as sequences that are complementary to the SMART oligonucleotide. Second-strand cDNA synthesis was accomplished by long distance PCR amplification of the first-strand cDNA using a specific SMART PCR primer according to instructions from the manufacturer. The SMART oligonucleotide, CDS primer, and PCR primer all contain a stretch of identical sequence. The final cDNA product was extracted with phenol-chloroform and then ethanol-precipitated and resuspended in 20 μl of water. Tester and driver cDNA samples were prepared in parallel at all times.

cDNA-RDA

Representational difference analysis was performed as described (Braun et al, 1995) with some modifications. Tester and driver cDNAs were digested with *Dpn*II (New England Biolabs, Beverly, Massachusetts), and for adaptor ligation or PCR, oligonucleotides used were as described (Hubank and Schatz, 1994). For the first cycle of subtractive hybridization, tester:driver ratios of 1:2 and 1:100 were simultaneously performed (for both type1 − type2 and type2 − type1). After hybridization for 20 hours at 67° C, the ends of the resulting hybrids were filled in, and a PCR amplification was done. The second and the third cycles of subtraction were performed similarly with ratios of 1:50 and 1:800 as well as 1:100 and 1:8000, respectively. Amplicons from the second or third rounds of subtractive hybridization were gel-purified by means of the QIAquick PCR purification kit (QIAGEN, Hilden, Germany), inserted into the pGEM-T Easy vector (Promega) at the *Eco*RV site, and transformed into *Escherichia coli* JM 109 cells.

Linker Capture Subtraction

Restriction enzyme digestion, linker ligation, and PCR amplification were done according to the method of Yang and Sytkowsky (1996) with minor modifications. Briefly, the double-stranded cDNA was digested with *Alu*I and *Rsa*I and then ligated to linkers that had a blunt end and a two-base 3′ protruding end, ACTCTTGCTTGGACGAGCTCT and ACTGAGAACGAACCTG CTCGAGA-p. The linker contained an *Alu*I/*Sac*I site near the blunt end. The top strand was designated the AP. The linker was prepared by combining an equal mass of each of the two oligodeoxynucleotides, heating the mixture to 90° C for 2 minutes, and allowing it to cool slowly to room temperature to enable anneal-

ing of the two strands. Ligation was carried out by mixing 1 μg of cut cDNA, 5 μg of linker, 1× ligation buffer (Stratagene, La Jolla, California) and 4 Weiss units of T4 DNA-ligase (Stratagene) in a volume of 10 μl, for 20 hours at 8° C. The reaction mixture was electrophoresed through a 2% low-melt agarose gel to remove unligated linkers, and linker-ligated cDNA fragments in the size range of 0.1–1.0 kb were collected. Linker-ligated cDNA fragments in agarose were amplified directly by PCR using the top strand sequence of the linker as the primer. The reaction (100 μl) contained 10 mM Tris-HCl, pH 8.9, 50 mM KCl, 0.1% Triton X-100, 200 μM dNTPs, 1μM AP primer, 2 mM $MgCl_2$, 1 μl of molten agarose, and 5 U *Taq* polymerase (Promega), running for 30 cycles (94° C, 1 minute; 55° C, 1 minute; 72° C, 1 minute). The amplified cDNA fragments were purified using a QIAEX purification kit (QIAGEN) and were used as the initial material for subtractive hybridization. After two rounds of subtraction, the PCR-amplified products were gel-purified, inserted into pGEM-T Easy vector (Promega), and transformed into *Escherichia coli* JM109 cells.

Dot Blot Hybridization and PCR and Sequence Analysis

Forty-eight white colonies from each subtracted cell type were picked at random and inoculated into LB + Ampicillin medium in individual wells of a 96-well plate. Two replica blots were prepared on Hybond™-N nylon filters using 25 μl of bacterial cells per well. The replica dot-blots were processed according to the method of Brown and Knudson (1991) and probed with random-primed–labeled driver DNAs from each fusion type.

Inserts were analyzed by direct PCR amplification of a 1 μl aliquot of each colony inoculum employing vector-specific primers (T7 and Sp6) for 30 cycles of 95° C, 1 minute; 46° C, 1 minute; and 72° C, 1 minute. The PCR products were electrophoresed on 2% agarose gels. The desired bands were excised and purified using the QIAquick PCR purification kit (QIAGEN). The products were then subjected to direct DNA sequencing and were employed to prepare probes for Northern blot analyses.

Northern Blotting and Phosphor-Imager Analysis

Ten μg of total RNA was fractionated on a 1.2% agarose/37% formaldehyde gel and transferred overnight onto a Hybond N membrane (Amersham, Aylesbury, United Kingdom) according to standard protocols. Prehybridization and hybridization reactions were performed at 42° C in 50% (v/v) formamide, 5 × SSC, 50 mM Tris-HCl, pH 7.5, 5 × Denhardt's solution, 5% SDS, and 250 μg/ml denatured salmon sperm DNA, and washed at a final stringency of 0.1 × SSC, 0.1% SDS at 55° C. Blots were hybridized to 32P-labeled cDNA probes and hybridization was detected by autoradiography. Quality and comparable loading of RNA samples were confirmed by including ethidium bromide in the gels and by rehybridizing blots to an 18S rDNA probe. After autoradiography, the filters were then exposed overnight to a Packard screen and scanned at 50 μm resolution in a phosphorimager instrument for quantification (Cyclone Instrument; Packard, Meriden, Connecticut).

RTQ-PCR Analysis of Clones

RTQ-PCR is based on the continuous optical monitoring of the progress of a fluorogenic PCR (Heid et al, 1996; Lo et al, 1999). The specificity of amplicon sequence selection was determined using primer and probe sequences that specifically detect the experimental gene sequence, as determined by means of the ABS module (Applied Biosysytems, Foster City, California). Analysis of gene expression was generated using an ABI Prism 7700 Sequence Detection System (TaqMan; Applied Biosystems, Foster City, California), which uses the 5′ nuclease activity of *Taq* DNA polymerase to generate a real-time quantitative DNA analysis assay (Gelmini et al, 1997; Heid et al, 1996). A nonextendable oligonucleotide hybridization probe with 5′ fluorescent and 3′ rhodamine (quench) moieties is present during the extension phase of the PCR. Degradation and release of the fluorescent moiety attributable to the 5′ nuclease activity results in peak emission at 518 nm and is monitored every 8.5 seconds by a sequence detector. The increase in fluorescence is monitored during the complete amplification process (real-time). A relative standard curve representing four-fold dilutions of stock cDNA of the gene of interest (1:2.5, 1:10, 1:40, and 1:160) was used for linear regression analysis of unknown samples. The expression of the housekeeping gene, GAPDH, was used to normalize for variances in input cDNA. The sequences of the PCR primer pairs and fluorogenic probe (5′ to 3′), respectively, that were used for each gene are as follows:

1S-3(VDAC): (forward) ccttggttgtgatgttgactttga, (reverse) gccctcataaccaaagacagct, and FAM-tttgctgg acctgcaatccatggtt-TAMRA

2-20(TAXREB107): (forward) ttacccccgggaccattct, (reverse) cttcaggaaaaccaccctcttg, and FAM-atcatcct cactggacgccacaggg-TAMRA

CAPN: (forward) cccacgcacacattactcca, (reverse) caaagagtctccggaactgcc, and TET-cattgaggccaacgag agtgaggagg-TAMRA

UBE1: (forward) gcaccacgtcaccagtactataacc, (reverse) aactgtttgagggtcatctcctca, and TET-agagtgga cattgtgggatcgctttga-TAMRA.

The fluorogenic probes are FAM, TET, and TAMRA.

Statistical Analyses

Duration of relapse-free survival (RFS) for patients with localized disease was computed from the date of diagnosis of EFT to the first occurrence of a disease event, defined as local or systemic relapse of EFT. Distributions of RFS were estimated according to the method of Kaplan and Meier, and comparisons of RFS were performed by means of log-rank tests. The Kruskal-Wallis analyses of variance was applied to test for differences in gene expression levels as mon-

itored by phosphorimaging of Northern blots or by RTQ-PCR.

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