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Membrane expression of MRP-1, but not MRP-1 splicing or Pgp expression, predicts survival in patients with ESFT

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Background: Primary Ewing's sarcoma family of tumours (ESFTs) may respond to chemotherapy, although many patients experience subsequent disease recurrence and relapse. The survival of ESFT cells following chemotherapy has been attributed to the development of resistant disease, possibly through the expression of ABC transporter proteins.

Methods: MRP-1 and Pgp mRNA and protein expression in primary ESFTs was determined by quantitative reverse-transcriptase PCR (RT-qPCR) and immunohistochemistry, respectively, and alternative splicing of MRP-1 by RT-PCR.

Results: We observed MRP-1 protein expression in 92% (43 out of 47) of primary ESFTs, and cell membrane MRP-1 was highly predictive of both overall survival ($P < 0.0001$) and event-free survival ($P < 0.0001$). Alternative splicing of MRP-1 was detected in primary ESFTs, although the pattern of splicing variants was not predictive of patient outcome, with the exception of loss of exon 9 in six patients, which predicted relapse ($P = 0.041$). Pgp protein was detected in 6% (38 out of 44) of primary ESFTs and was not associated with patient survival.

Conclusion: For the first time we have established that cell membrane expression of MRP-1 or loss of exon 9 is predictive of outcome but not the number of splicing events or expression of Pgp, and both may be valuable factors for the stratification of patients for more intensive therapy.

Current response rates of Ewing's sarcoma family of tumour (ESFT) patients to first-line chemotherapy are as high as 77% (Bacci *et al*, 2000; Yonemori *et al*, 2007; Gaspar *et al*, 2012; Serlo *et al*, 2012), demonstrating that some ESFTs are chemoresponsive. However, others are resistant to chemotherapy at diagnosis (23%), and this is known as intrinsic resistance. Despite the high response rate, 20–40% of patients experience disease recurrence (Ahrens *et al*, 1999; Cotterill *et al*, 2000; Rodriguez-Galindo *et al*, 2007; Stahl *et al*, 2011) and only 10–23% are alive at 5 years (Barker *et al*, 2005; Huang and Lucas, 2011; Stahl *et al*, 2011). The survival of drug-resistant clones after initial chemotherapy and the high occurrence of relapse have been attributed to the development of acquired drug resistance. The ability to evade cell death in this way

represents a major challenge for improving patient outcome and survival.

Several mechanisms have been implicated in the development of resistance, including the efflux of agents (Seidel *et al*, 1995; Panyam and Labhasetwar, 2003) by multidrug resistance (MDR) ABC transporter proteins (Oda *et al*, 1997), in particular the over-expression of Pgp and MRP-1. Increased expression of these efflux proteins results in the removal of chemotherapeutic agents from the cell, reduced effectiveness of chemotherapeutics and decreased survival (Burger *et al*, 2003). To date, Pgp is the only MDR protein to have been investigated in ESFTs. However, the results of published studies are contradictory, one suggesting that Pgp was not predictive of prognosis (Perri *et al*, 2001) and a second

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significantly linking expression of Pgp to poor response to chemotherapy (Roessner *et al*, 1993).

Therefore, in this study we have investigated the hypothesis that intrinsic drug resistance in primary ESFTs is mediated through the expression of the ABC transporter proteins Pgp and MRP-1, and that these may be predictive of patient outcome and response to therapy. As functional splice variants of MRP-1 have been described in some normal and cancer cells (Cole *et al*, 1992; Grant *et al*, 1997; He *et al*, 2004), we have also examined for the first time whether this arises in primary ESFTs, and whether the pattern of spliced variants might be prognostic.

MATERIALS AND METHODS

Patients and clinical data. Forty-nine cases with a confirmed diagnosis of primary ESFTs, made by independent pathology review and by reverse-transcriptase PCR (RT-PCR) detection of the *EWS-ETS* gene rearrangement or CD99 positive by immunohistochemistry (IHC), were included in the study (Table 3). Clinical data were provided by the Children's Cancer and Leukaemia Group Data Centre, University of Leicester. Information was sought on the diagnosis date, date of relapse or death and current disease status. Patient age at diagnosis ranged from 2 to 38 years, with a median age of 13 years, and patients were 22 men and 25 women. Tumours were collected from 1993 to 2006, mounted in optimum cutting temperature (OCT) and stored in liquid nitrogen. Informed consent and ethical approval was obtained for the use of frozen tumour material in these biological studies (MREC 98/4/023, dated 24 July 2001).

Control cell lines and tissues. The substrate-adherent Ewing's sarcoma TTC 466 and the neuroblastoma (NB) SK-N-SH cell lines were obtained and cultured as previously described (Roundhill and Burchill, 2012). Both cell lines are yeast-, bacterial- and mycoplasma-free and are determined every 4 months using the EZ-PCR mycoplasma test kit (Geneflow, Lichfield, UK). The breast adenocarcinoma was obtained from a local anonymised pathology tissue archive (LREC approval, dated 17 July 2001).

Immunohistochemistry for MRP-1 and Pgp in primary ESFTs. Immunohistochemistry for MRP-1 expression was performed on 47 tumours (Dalal *et al*, 2005). Sections (5 μ m), fixed using methanol:acetone (1:1; Merck Biosciences, San Diego, CA, USA) for 2 $\times$ 2 min at room temperature on glass slides, were incubated with a polyclonal MRP-1 antibody (20 μ g ml⁻¹; A23, Axxora, Exeter, UK) and diluted in antibody diluent (DakoCytomation, Invitrogen, Paisley, UK) for 1 h, followed by an incubation with a secondary antibody (1 μ g ml⁻¹, goat anti-rabbit; DakoCytomation; diluted in antibody diluent) for 30 min, both at room temperature. Breast adenocarcinoma was used as a positive control for MRP-1 expression; optimum antibody concentration was determined empirically.

Pgp expression was detected in acetone-fixed (4 °C for 10 min) sections (5 μ m) by using the EnVision + System-HRP (DAB) kit (DakoCytomation). Sections were incubated with Pgp antibody (10 μ g μ l⁻¹; JSB-1, Axxora) and were diluted in antibody diluent overnight at 4 °C. The SK-N-SH NB cell sections (5 μ m), mounted in OCT compound, were used as a positive control for Pgp expression; optimum antibody concentration was determined empirically.

All sections were visualised using the Liquid DAB Substrate Chromogen System for peroxidase (DakoCytomation) and were counterstained with haematoxylin. Sections were visualised by light microscopy (Zeiss Axioplan microscope; Zeiss, Cambridge, UK). MRP-1 and Pgp expression were scored manually by two independent reviewers, using light microscopy to visualise the sections; each reviewer was blind to the clinical outcome data.

MRP-1 expression was scored as the number of positively stained cells out of 100 in 4 randomly selected areas of each tumour; the number of cells with membrane localisation was also scored. Cells were scored as positive or negative for Pgp.

Extraction of DNA and RNA. Frozen primary ESFT were stained with haematoxylin, ESFT cells captured by laser capture microdissection (LCM). RNA and DNA from the cell lines were extracted as previously described, and quality and quantity were examined (Brownhill *et al*, 2007).

Quantitative RT-PCR for MRP-1 and Pgp. For the analysis of ABC transporter mRNA expression by quantitative RT-PCR (qRT-PCR), 10 ng or 1 ng per 1 μ g of cell line RNA was amplified for MRP-1 and Pgp, respectively; 5 ng of RNA from LCM primary tumour was amplified for MRP-1 and Pgp. Total RNA was reverse transcribed using Superscript III Reverse Transcriptase (Invitrogen; Roundhill and Burchill, 2012). Samples were analysed in triplicate and in the absence of RT enzyme or without sample RNA (replaced with DNase-/RNase-free H₂O; Invitrogen), to control for non-specific amplification of DNA and contamination, respectively.

Complementary DNA was added to a PCR mix containing sequence-specific reverse and forward primers (Supplementary Figure 1) and 1 $\times$ TaqMan Universal PCR Master Mix (Invitrogen). Samples were denatured for 2 min at 50 °C, Taq polymerase was activated by heating for 10 min at 95 °C and cDNA amplified using 40 cycles of 95 °C for 15 s and 60 °C for 1 min. mRNA expression was calculated using the comparative Ct method (Brownhill *et al*, 2007), relative to the housekeeping genes β -2-microglobulin (Brownhill *et al*, 2007) and peptidylprolyl isomerase A (PPIA) (Fischer *et al*, 2005; Lastowska *et al*, 2007), in cell lines (Supplementary Figure 2) and primary ESFTs, respectively. TTC 466 and SK-N-SH cell line RNA was included as a reference control for interassay variability in the amplification of MRP-1 and Pgp, respectively.

Identification of MRP-1 pre-RNA splicing. Total RNA (500 ng or 10 ng from cell lines (Supplementary Figure 2) and primary ESFTs, respectively) was reverse transcribed as previously described (Roundhill and Burchill, 2012) and cDNA added to a PCR mix containing MRP-1 exon-specific primers (Supplementary Figure 1) and 1 unit of AmpliTaq Gold DNA polymerase in 1 $\times$ AmpliTaq Reaction Buffer II (Invitrogen). The AmpliTaq gold was activated by one cycle at 95 °C for 10 min and cDNA amplified for 35 cycles of 95 °C for 30 s, 60 °C for 30 s and extension at 72 °C for 1 min. This was followed by a further final extension at 72 °C for 10 min. PCR products were separated by 2% agarose gel electrophoresis and were visualised after staining with ethidium bromide (0.5 μ g ml⁻¹; Sigma-Aldrich Company Ltd., Dorset, UK), under UV light.

Statistical analysis. The prognostic value of MRP-1 and Pgp expression, and one or more MRP-1 splicing events, was evaluated using the Cox proportional hazards model; results with *P*-values <0.05 were considered significant. Overall survival (OS) was calculated as the time from diagnosis to the date last seen, regardless of the number of events that may have occurred. Event-free survival was defined as the time from diagnosis to the time of first event; the first event could be relapse, death or date last seen for surviving patients. Patients without time to a first event or OS follow-up data were excluded from analyses; clinical follow-up information was available in 33 out of 38, 39 out of 44 and 44 out of 47 patients for alternative splicing, qRT-PCR and IHC, respectively. There was limited tumour tissue available from some patients and, hence, it was not possible to analyse all tumours for alternative splicing, protein and mRNA. The statistical methodology used to identify the optimal cut-point to maximise the difference in OS and time to a first event between patient groups

with high and low MRP-1 expression is as previously described (London *et al*, 2005). Briefly, each decile of MRP-1 expression was evaluated for a statistically significant difference in OS and time to a first event. Specific associations between RNA and protein levels, and between individual splicing events, were determined by Spearman's rank and Pearson's correlations, respectively.

RESULTS

MRP-1 is more frequently expressed in primary ESFTs than in Pgp; the level of cell membrane MRP-1 expression predicts patient outcome. MRP-1 was expressed in 91% (43 out of 47) of the primary ESFTs analysed (Figures 1A and B); staining was absent in 4 out of 47 ESFTs (Figure 1C). The pattern of MRP-1 expression was similar across each tumour sample. Samples either expressed intracellular MRP-1 only (which was cytoplasmic and/or nuclear, 5 out of 43; Figure 1A), or both intracellular and membrane-localised MRP-1 (38 out of 43; Figure 1B). No tumour expressed MRP-1 in the membrane alone. MRP-1 total protein (MRP-1^T) or membrane protein (MRP-1^{Mem}) expression was not predictive of response to therapy measured by necrosis (MRP-1^T, $P=0.34$; MRP-1^{Mem}, $P=0.09$). However, MRP-1^{Mem} but not MRP-1^T was predictive of time to a first event (MRP-1^T, $P=0.096$; MRP-1^{Mem}, $P<0.0001$; cut-off upper 10% high expressers; Figure 1D) and OS (MRP-1^T, $P=0.75$; MRP-1^{Mem}, $P<0.0001$; cut-off upper 10% high expressers; Figure 1E) in these patients.

All mRNAs from primary ESFTs (44) successfully amplified for the endogenous control gene PPIA (mean Ct = 25, range = 21–30), confirming the quality of the tumour RNA. Expression of MRP-1 mRNA was heterogeneous in primary ESFTs and was detected in 82% (36 out of 44) of tumours (Figure 2A; mean Ct = 33, range 30–39). MRP-1 mRNA was detected in three out of four MRP-1-negative tumours. The level of MRP-1 mRNA was not predictive of

response to therapy measured by necrosis ($P=0.82$) but was a predictor of time to a first event ($P=0.03$, 0.15; cut-off upper 20% and 10% high expressers, respectively; Figure 2B), but not OS ($P=0.1$; cut-off upper 10% high expressers; Figure 2C). There was no significant correlation between MRP-1 mRNA and MRP-1^T ($r=0.11$) or MRP-1^{Mem} ($r=0.06$) expression.

Pgp protein expression was identified in only 6% (3 out of 47) of the primary ESFTs analysed (Figures 3A and B), where expression was observed in the cell membrane and cytoplasm. Pgp was not expressed in the nucleus. Pgp protein expression was not predictive of patient response to treatment measured by necrosis ($P=0.64$), time to a first event ($P=0.60$) or OS ($P=0.28$). In contrast, Pgp mRNA was detected in 86% (38 out of 44) of tumour samples analysed (Figure 3C; mean Ct = 34, range = 30–39). The level of Pgp mRNA was not predictive of response to therapy measured by necrosis ($P=0.22$), time to a first event ($P=0.25$; cut-off upper 10% high expressers) or OS ($P=0.20$; cut-off upper 10% high expressers). Similar to MRP-1, there was no correlation between Pgp mRNA and protein expression ($P=0.81$).

A cohort of primary ESFTs were analysed for both MRP-1 and Pgp, mRNA and protein. There was no correlation between the Pgp protein expression and membrane ($P=0.97$) or total ($P=0.64$) MRP-1 expression. However, there was a significant correlation between the levels of Pgp and MRP-1 mRNA ($P=0.026$).

MRP-1 pre-mRNA is alternatively spliced in primary ESFTs. Alternative splicing of MRP-1 was observed in 63% (24 out of 38) of tumours (Figure 4 and Table 1). In all MRP-1 protein-positive samples, the full-length PCR product was amplified (Figure 4 and Table 1). The statistically significant paired exon losses (e.g., exon 5 and 6 together) are summarised in Table 2.

There is no correlation between the number of splicing events and the MRP-1 mRNA by RT-PCR ($r^2=0.1378$, $P=0.4159$) and

Figure 1. MRP-1 protein expression in primary ESFTs. Positive MRP-1 protein expression was (A) intracellular or (B) localised to the membrane and intracellular ($n=45$). (C) Some primary ESFT expressed no MRP-1 ($n=4$). N = nuclear; M = cell membrane MRP-1 staining. The relationship between cell membrane MRP-1 and (D) time to a first event or (E) OS was analysed by the Cox proportional hazards model ($n=44$). Grey dotted line = 10% of tumours expressing MRP-1; blue solid line = remaining patients (lower 90% of tumours expressing MRP-1); n = number of tumours analysed.

Figure 2. MRP-1 mRNA expression in primary ESFTs. **(A)** There was heterogeneous mRNA expression of MRP-1 in primary ESFT. MRP-1 mRNA expression was calculated using the comparative Ct method, relative to the housekeeping gene PPIA and the reference sample, the ESFT cell line TTC 466 ($n = 46$); data presented as $\Delta\Delta C_t$. The relationship between mRNA expression and **(B)** time to a first event or **(C)** OS was analysed by the Cox proportional hazards model ($n = 39$). Grey dotted line = 10% of tumours expressing MRP-1; blue solid line = remaining patients (lower 90% of tumours expressing MRP-1); n = number of tumours analysed.

Figure 3. Pgp expression in primary ESFTs. **(A)** Positive Pgp expression was observed in the cell membrane and cytoplasm of cells ($n = 3$ tumours). **(B)** The majority of primary ESFTs were negative for Pgp expression ($n = 46$). C = cytoplasmic and M = cell membrane Pgp staining. **(C)** There was heterogeneous mRNA expression of Pgp in primary ESFT. Pgp mRNA expression was calculated using the comparative Ct method, relative to the housekeeping gene PPIA and the reference sample, the NB cell line SK-N-SH; data presented as $\Delta\Delta C_t$; n = number of tumours analysed.

MRP-1^{MEM} expression ($r^2 = 0.3070$, $P = 0.0646$) in primary ESFTs. However, MRP-1^{MEM} and mRNA expression were significantly associated with loss of exons 5 or 6 ($r^2 = 0.3440$, $P = 0.0371$) and

exon 17 ($r^2 = 0.3551$, $P = 0.0336$), respectively, suggesting that in conditions of high MRP-1 expression pre-RNA splicing may increase.

Figure 4. MRP-1 splicing events observed in ESFT primary tissues. Splicing events observed in primary ESFTs (sample 40) generated by RT-PCR, employing 11 primer mixes spanning the 31 exons of MRP-1 (Supplementary Figure 1). Image shows ethidium bromide-stained amplified products after separation by electrophoresis and visualisation under UV light. * = splice variant; ← = full-length/predicted PCR product; ladder = 50 bp DNA ladder; + = RT-positive amplified sample; – = RT-negative control; + H₂O = control for contamination containing RT, but in which RNA is replaced with H₂O; H₂O = negative control in which cDNA is replaced with H₂O in PCR step.

Although the loss of exons 6, 9, 23 and 29, which was frequently observed in this sample group, resulted in a frame shift (Table 1), which we anticipate would produce a non-functional protein, there is no correlation between time to a first event, OS and the number of or a particular splicing event ($P > 0.05$). However, interestingly, patient relapse was significantly associated with loss of exon 9 ($P = 0.041$) (Table 2).

DISCUSSION

Cell membrane expression of MRP-1 in ESFTs at diagnosis strongly predicts for time to a first event and OS, consistent with the hypothesis that cell membrane MRP-1 is in part responsible for drug resistance in ESFT. This is the first study of total and membrane MRP-1 expression and prognostic value in primary ESFTs. MRP-1 protein was expressed in the nucleus and cell membrane of primary ESFTs, consistent with reports in other cancer types (Izquierdo *et al*, 1995; Maraldi *et al*, 1999; Molinari *et al*, 2002). Total MRP-1 expression did not correlate with time to first event, OS or necrosis, which is in agreement with previous studies evaluating total MRP-1 expression in paediatric and adult cancers (Izquierdo *et al*, 1995; Filipits *et al*, 1997; Styczynski *et al*, 2007). Similar to the current study, MRP-1 mRNA expression has been associated with time to a first event and OS in NB (Norris *et al*, 1996; Haber *et al*, 2006) and a panel of other childhood malignancies (Plasschaert *et al*, 2005; Schaich *et al*, 2005). However, in adult cancers, such as breast and ovarian carcinomas, MRP-1 mRNA expression was not predictive of time to a first event (Izquierdo *et al*, 1995; Burger *et al*, 2003), OS (Plasschaert *et al*, 2005) or necrosis (Burger *et al*, 2003), suggesting that MRP-1 may be more important in cancers of young people than in adult disease. Whether this reflects the developmental origin of the cancers requires further investigation.

Pgp was expressed in only 3 out of 47 primary ESFTs; whether this reflects methylation of the *MDR1* gene as in ESFT cell lines (Supplementary Figure 2) remains to be seen. In agreement with the current work, Perri *et al* (2001) did not observe a correlation between Pgp protein expression and time to a first event ($P = 0.87$) or OS ($P = 0.79$) in patients at diagnosis. However, protein expression of Pgp has been associated with poor response to therapy ($P < 0.08$; Roessner *et al*, 1993) in pre- and post-diagnosis ESFTs. Unfortunately, in this study the authors failed to distinguish between pre- and post-treatment groups, which might explain the difference in study conclusions. Increased Pgp protein expression has however been linked to an adverse OS in other paediatric (Baldini *et al*, 1995; Filipits *et al*, 1997) and adult cancers (Mignogna *et al*, 2006). We have also shown that Pgp mRNA expression was not predictive of patient outcome, in agreement with previous studies in children with NB (Norris *et al*, 1996;

Haber *et al*, 1997). In contrast, Pgp mRNA expression in some paediatric and adult cancers taken at diagnosis has been associated with a worse OS (Schaich *et al*, 2005), time to a first event (Burger *et al*, 2003; Kourti *et al*, 2007) and response to treatment (Burger *et al*, 2003). As Pgp protein was expressed in only 3 out of 47 tumours, we agree it is difficult to conclude on the prognostic value of Pgp in the current study cohort. What we can conclude is that MRP-1 is the dominant predictive MDR protein in ESFTs taken at diagnosis. Whether Pgp is of additional predictive value at diagnosis in a small group of patients or has a role in drug-induced resistance remains to be seen.

Although significant correlations between the mRNA and protein expression of MDR proteins have been described (Nooter *et al*, 1995; Cai *et al*, 1999; Yang *et al*, 2002; Lacueva *et al*, 2005; Haber *et al*, 2006), in the current study there was no correlation between Pgp protein and mRNA, or full and membrane MRP-1 protein and mRNA in primary ESFTs. These MDR proteins are highly posttranscriptionally regulated by processes such as methylation (Kantharidis *et al*, 1997; Ando *et al*, 2000; Qiu *et al*, 2007). This is supported by the regulation of Pgp expression in cell lines by methylation of *MDR1* (Supplementary Figure 2). No correlation was observed between total MRP-1 and Pgp protein expression, consistent with post-transcriptional regulation of the two proteins (Hipfner *et al*, 1997; Zhou, 2008). In agreement with previous studies (Almquist *et al*, 1995; Nooter *et al*, 1995; Lu *et al*, 2004), we observed a significant correlation between MRP-1 and Pgp mRNA, possibly highlighting a similar mechanism of transcriptional regulation. Interestingly, decreased expression of miR-326 and miR-451 has been associated with increased MRP-1 and Pgp expression, respectively, regulating gene transcription through the 3'-UTR of the ABC transporter proteins (Kutanzi *et al*, 2011). In addition, the chromosome rearrangement EWS-FLI1 (Burchill, 2003) has been shown to increase EYA3 through miR-708 (Robin *et al*, 2012). We are currently investigating the role of micro-RNAs in the development of MDR.

In this study, we have described for the first time the presence of MRP-1 splice variants in primary ESFTs. We found loss of exon 9 was predictive of patient relapse (five out of six patients remained in continuous complete remission) and so represents a prognostic marker that requires further investigation. Although splicing of MRP-1 has not previously been correlated with patient outcome, alternative splicing of P73 has been identified as a negative prognostic marker in patients with NB (Romani *et al*, 2003). In contrast, upregulation of the alternative MDM4 and MDM2 splice variants, described in soft tissue sarcomas (Bartel *et al*, 2005) and breast cancer patients (Lukas *et al*, 2001), have both been correlated with poor OS (Lukas *et al*, 2001; Bartel *et al*, 2005). Alternative splicing of exons 10–19 and exons 5, 13, 17, 18 and 30 in addition to WT MRP-1 expression, has previously been described in a range of cell types (Grant *et al*, 1997) and ovarian cancers (He *et al*, 2004), respectively. We also identified pre-RNA

Table 1. Summary of the splicing events observed in all primary ESFTs evaluated

Tumour	Splicing	Exon number																															Number
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	
1																																	8
2																																	11
3																																	7
4																																	9
5																																	0
7																																	0
8																																	2
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29																																	7
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31																																	4
32																																	0
33																																	10
35																																	2
36																																	2
37																																	0
38																																	0
40																																	0
41																																	0
42																																	0
43																																	0
44																																	0
46																																	2
49																																	6
Frame						F			F					F	F	F			F	F	F	F	F	F	F	F			F	F	N/A		

Abbreviations: Black=splicing; ESFT=Ewing's sarcoma family of tumour; F=loss of this exon would cause a frame shift; grey=no MRP-1 protein expression by IHC; IHC=immunohistochemistry; number=total number of splicing events in the sample; samples 1-49=primary ESFTs; white=no splicing.

splicing of MRP-1 in both cancer and normal cell lines (Supplementary Figure 2), suggesting that splicing of MRP-1 is not a cancer-specific phenomenon. Interestingly, the splicing of 10 specific exons occurred at a significantly higher frequency in cell lines (Supplementary Figure 2) than in primary ESFTs, most likely reflecting changes in MRP-1 splicing following the adaptation of cells *in vitro*. Loss of exons 3, 5, 17 and 18 result in the loss of whole amino acids and loss of exons 6, 9, 23 and 29 each result in a frame shift, all of which have been observed in this study and are likely to produce a non-functional protein. In agreement with this, MRP-1 splice variants missing each of exon groups 12-26, 17 and 18, 5 and 30 were reported to all confer drug resistance in HEK293T cells when overexpressed, although the effect was not as significant as that of the full-length MRP-1 (He *et al*, 2004).

Although in this study we have not investigated the regulation of alternative splicing, overexpression of the splicing factors PTB and SRp20 (He *et al*, 2004) have been linked to increased MRP-1 splicing. Furthermore, the level of *trans*-acting and *cis*-elements, and the effects of external stimuli, have been reported to determine the accuracy of pre-mRNA splicing (Maniatis and Tasic, 2002), although the mechanism controlling these are not yet understood. Although some studies have suggested that EWS-ETS fusion proteins (Burchill, 2003) inhibit splicing of pre-mRNA (Yang *et al*, 2000a,b), EWS-FLI1 specifically has been shown to directly interact with the splicing factors U1C and SF1 (Knoop and Baker, 2001), and has been reported to increase transcript levels of cyclinD1b in ESFT cells through alternative splicing (Sanchez *et al*, 2008). Whether this is the case for MRP-1 in ESFT cells requires further investigation.

In addition to full-length MRP-1 protein, we have also identified novel shortened MRP-1 protein products (MRP-1-100); products of 65-190 kDa have been reported in a range of other cancer types (Krishnamachary *et al*, 1994; Regina *et al*, 1998; Nuti *et al*, 2000; Fernetti *et al*, 2001). As we have observed splicing of the MRP-1 pre-mRNA in ESFT cell lines, it is possible that MRP-1-100 represents a protein product of alternative splicing, although this requires further investigation. Interestingly, alternatively spliced pre-mRNA transcripts have been described in other members of the ABC transporter family, such as Pgp (Devine *et al*, 1991), BCRP (Nakanishi *et al*, 2006), ABCG1 (Engel *et al*, 2006) and ABCA1 (Bocchi *et al*, 2010), and have been associated with a change in protein activity, suggesting that abnormal splicing may represent an additional mechanism of ABC transporter expression control.

Although a variety of other mechanisms of MDR have been described in ESFTs, including defective DNA repair (Alldinger *et al*, 2007), overexpression of anti-apoptotic proteins (Batra *et al*, 2003), glutathione metabolism (Scotlandi *et al*, 2009) and the presence of cancer-initiating cells (Suva *et al*, 2009), the results of this study suggest that targeting the ABC transporter protein MRP-1 may be important to improve the response to therapy and survival for relapsed ESFT patients. High MRP-1 cell membrane protein expression might be useful to identify those patients that are most likely to benefit from more aggressive treatment, combination treatment with standard chemotherapeutics and MRP-1 inhibitors, or treatment with a non-MRP-1 substrate (Roundhill and Burchill, 2012). Similarly, patients with low MRP-1 or loss of MRP-1 exon 9 could be spared unnecessary toxicity, while decreasing the cost of treatment. Before any clinical benefit can be realised, it is now important to validate cell membrane

Table 2. A correlation matrix, calculated by Pearson's correlation, evaluating the association between the loss of specific exons in 40 primary ESFTs

Exon number	3	5	6	8	9	13	17	18	23	24	26	27
5	$r^2 = 0.4416$ $P = 0.0043$											
6	$r^2 = 0.4416$ $P = 0.0043$	$r^2 = 1$ $P = 0$										
13	$r^2 = 0.3977$ $P = 0.011$	$r^2 = 0.5041$ $P = 0.0009$	$r^2 = 0.5041$ $P = 0.0009$		$r^2 = 0.3977$ $P = 0.011$							
17	$r^2 = 0.319$ $P = 0.0485$	$r^2 = 0.4841$ $P = 0.0018$	$r^2 = 0.4841$ $P = 0.0018$			$r^2 = 0.4866$ $P = 0.0017$						
18	$r^2 = 0.3977$ $P = 0.011$	$r^2 = 0.5041$ $P = 0.0009$	$r^2 = 0.5041$ $P = 0.0009$			$r^2 = 0.5733$ $P = 0.0001$	$r^2 = 0.8083$ $P = 0$					
23					$r^2 = 0.4120$ $P = 0.0082$	$r^2 = 0.3676$ $P = 0.0196$		$r^2 = 0.3676$ $P = 0.0196$				
24		$r^2 = 0.3974$ $P = 0.0111$	$r^2 = 0.3974$ $P = 0.0111$		$r^2 = 0.4763$ $P = 0.0019$	$r^2 = 0.3318$ $P = 0.0365$		$r^2 = 0.488$ $P = 0.0014$	$r^2 = 0.7534$ $P = 0$			
26					$r^2 = 0.4120$ $P = 0.0082$	$r^2 = 0.3676$ $P = 0.0196$		$r^2 = 0.3676$ $P = 0.0196$	$r^2 = 0.6376$ $P = 0$	$r^2 = 0.4664$ $P = 0.0024$		
27		$r^2 = 0.3504$ $P = 0.0266$	$r^2 = 0.3504$ $P = 0.0266$	$r^2 = 0.4804$ $P = 0.0017$	$r^2 = 0.3267$ $P = 0.0396$	$r^2 = 0.4303$ $P = 0.0056$				$r^2 = 0.3780$ $P = 0.0162$	$r^2 = 0.5379$ $P = 0.0003$	
28					$r^2 = 0.4120$ $P = 0.0082$			$r^2 = 0.3676$ $P = 0.0196$		$r^2 = 0.7534$ $P = 0$		
29	$r^2 = 0.3267$ $P = 0.0396$	$r^2 = 0.3504$ $P = 0.0266$	$r^2 = 0.3504$ $P = 0.0266$	$r^2 = 0.480$ $P = 0.0017$								$r^2 = 0.4444$ $P = 0.0041$

Abbreviations: ESFT = Ewing's sarcoma family of tumour.

Table 3. Clinical patient data on histologically confirmed ESFTs

Tumour number	Age at diagnosis	Primary tumour site	Tumour volume (ml)	Metastasis (including pulmonary, bone, bone marrow) at diagnosis (1 = metastasis, 0 = no metastasis)	Response to therapy	Current disease status score (1 = died of disease, 0 = alive/alive with disease)	Follow-up time (days)	Time to first event (days)	Relapse score (1 = relapse, 0 = no relapse)	EWS-ETS gene fusion type
1	12					0	1597	1449	1	EWS-FLI1 type 2
2	12	Right foot	<200	1	60	0	4536	4536	0	EWS-FLI1 type 1
3	2	Pelvis buttock SCG				0	2497	2497	0	EWS-ERG
4	14	Femur		1	100	0	1519	1519	0	EWS-FLI1 type 2
5	13	Right fibula	40	1	80	0	1827	1436	1	EWS-FLI1 type 2
6					100					EWS-FLI1 type 1
7	15	Tibia	134	0	100	0	2040			EWS-FLI1 type 1
8	14	Rib	<200	0		1	2575	1290	1	EWS-FLI1 type 2
9	11	Humerus			100	0	1164			EWS-FLI1 type 1
10										EWS-FLI1 type 1
11	14	Rib	816	1	100	0	2238	2238	0	EWS-FLI1 type 1
12	12	Scapula	420	1		1	408	309	1	EWS-ERG
13	19	Left ilium		0		1	641	492	1	EWS-FLI1 other
15	11	Ankle, foot, heel, toe, bones	275	0	65	0	2095	2095	0	EWS-FLI1 type 1
16	10	Pelvis: acetabulum				0	1294	1294	0	EWS-FLI1 type 1
17	13	Iliac				0	1253	1253	0	EWS-FLI1 type 1
18	25	Left ilium		0		0	126	126	0	EWS-FLI1 other
19	14	Fibula	67	1	99	0	3181	3181	0	EWS-FLI1 type 1
20	2	Pelvis/sacrococcygeal				0	1731	1731	0	EWS-FLI1 type 2
21	10	Thorax: chest wall	> 200	0	95	0	1989	1989	0	EWS-FLI1 other
22	2	Pelvis/sacrococcygeal					1732	1732	0	EWS-FLI1 type 2
23	6	Right distal femur	58	1		0	307	307	0	EWS-FLI1 type 2
24	16	Right proximal femur	1640	1	99	0	2222	2222	0	EWS-FLI1 type 1
25	8	Thorax: chest wall	> 200	0	95	1	430	296	1	EWS-FLI1 type 1
26	14	Right heel	147	1	100	0	2619	2619	0	NA, CD99 positive
27	15	Chest wall, rib involvement	242	0	100	0	2655	2655	0	NA, CD99 positive
28	14	Left femur		1	100	0	3605	3605	0	EWS-FLI1 other

Table 3. (Continued)

Tumour number	Age at diagnosis	Primary tumour site	Tumour volume (ml)	Metastasis (including pulmonary, bone, bone marrow) at diagnosis (1 = metastasis, 0 = no metastasis)	Response to therapy	Current disease status score (1 = died of disease, 0 = alive with disease)	Follow-up time (days)	Time to first event (days)	Relapse score (1 = relapse, 0 = no relapse)	EWS-ETS gene fusion type
29	10	Right posterior thoracic wall	50.6	0		0	225	225	0	NA, CD99 positive
30	9	Rib	300	0	100	0	3238	3238	0	EWS-FLI1 type 1
31	10	Chest wall	992.25	0	30	1	197	115	1	EWS-FLI1 type 1
32	15	Buttock	1485	1		1	714	397	1	NA, CD99 positive
33	7	Spinal cord - cervical cord	<200	0	100	0	1965	1965	0	EWS-FLI1 type 2
35	13	Right Calcaneum	211	1	100	0	2455	634	1	EWS-FLI1 type 1
36	14	Right iliac wing		0		0	1564	972	1	EWS-FLI1 type 1
37	12	Pelvis/sacrococcygeal								EWS-FLI1 type 1
38	4	Right paravertebra	660	1	<50	1	371	252	1	EWS-FLI1 type 1 and EWS-ERG
39	9	Rib		0	100	1	1092	1037		EWS-FLI1 type 1
40	14	Rib	344	0	20	0	1624	1624	0	NA, CD99 positive
41	9	Right retroperitoneal	42	0		0	1931	1931	0	EWS-FLI1 type 1
42	15	Ischiopubic bone	353	0	100	1	952	325	1	EWS-FLI1 type 1
43	13	Lower limb - hip				0	1226		0	EWS-FLI1 type 1
44	15	Right pubis	152	0	>90	0	2579	2579	0	EWS-FLI1 type 1
45	8	Femur		1	90	1	434	586		EWS-FLI1 type 1
46	18	Distal tibia	217	0	20	0	2154	2154	0	EWS-FLI1 type 1
47	16	Iliac bone		0			952			EWS-FLI1 type 1
48	13	Pelvis/iliac fossa		1		1	700	469	1	EWS-FLI1 type 1
49	11	Buttock	55	0		0	2523	2523	0	EWS-FLI1 type 2
50	16	Left iliac crest	794	1		1	366	366	0	EWS-FLI1 type 1
51	22	Right thigh		0	<100	1				EWS-FLI1 type 1
52	10	Maxilla	>200	0	100	0	2036	2036	0	EWS-ERG
53	24	Left distal humerus		0	>90	0	1892	1892	0	EWS-FLI1 type 1
54	38	Left thigh	>200	0	<100	0	1448	1110	1	NA, CD99 positive
55	12	Fibula	157	0	50	1	666	508	1	EWS-FLI1 type 1

Abbreviations: ESFT = Ewing's sarcoma family of tumour, NA = EWS-ETS gene status not available; SCG = sacrococcygeal presacral. Tumours 6, 10, 37, 51 were not included in survival analysis as there was no available patient data, but were incorporated into analyses evaluating the frequency of protein, mRNA and splicing events.

MRP-1 and loss of exon 9 as a predictive marker in ESFTs, and to determine the independent clinical significance of these findings.

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