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Osteosarcoma is characterised by reduced expression of markers of osteoclastogenesis and antigen presentation compared with normal bone

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BACKGROUND: Osteosarcoma (OS) is the most common primary bone tumour in children and adolescents. Patients who respond poorly to chemotherapy have a higher risk of metastatic disease and 5-year survival rates of only 10–20%. Therefore, identifying molecular targets that are specific for OS, or more specifically, metastatic OS, will be critical to the development of new treatment strategies to improve patient outcomes.

METHODS: We performed a transcriptomic analysis of chemo-naive OS biopsies and non-malignant bone biopsies to identify differentially expressed genes specific to OS, which could provide insight into OS biology and chemoresistance.

RESULTS: Statistical analysis of the OS transcriptomes found differential expression of several metallothionein family members, as well as deregulation of genes involved in antigen presentation. Tumours also exhibited significantly increased expression of ID1 and profound down-regulation of S100A8, highlighting their potential as therapeutic targets for OS. Finally, we found a significant correlation between OS and impaired osteoclastogenesis and antigen-presenting activity. The reduced osteoclastogenesis and antigen-presenting activity were more profound in the chemoresistant OS samples.

CONCLUSION: Our results indicate that OS displays gene signatures consistent with decreased antigen-presenting activity, enhanced chemoresistance, and impaired osteoclastogenesis. Moreover, these alterations are more pronounced in chemoresistant OS tumour samples.

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Osteosarcoma (OS) is the most common primary malignant bone tumour in children and adolescents (Chou and Gorlick, 2006). Disease progression is marked by aggressive growth, local recurrence, and poor long-term survival rates because of the development of fatal pulmonary metastasis in up to 50% of patients (Wang, 2005). Chemotherapy over many weeks results in disease-free survival rates of 50-70% for patients with non-metastatic disease (Bacci et al, 1997; Marina et al, 2004). However, patients who have a poor response to chemotherapy have a higher risk of developing pulmonary metastases, which results in survival rates of <20% (Chou and Gorlick, 2006). To develop new-targeted therapies to treat OS will require knowledge of the specific defects associated with non-metastatic and metastatic disease. Tests to predict whether a patient will respond to current chemotherapeutics or develop metastases would enhance our ability to select appropriate treatment strategies. To this end, a number of studies of OS have generated gene expression signatures, which have provided insights into OS biology (Leonard et al, 2003; Baird et al, 2005) and chemoresistance (Ochi et al, 2004; Man et al, 2005; Mintz et al, 2005). However, these studies have focused on comparisons of chemosensitive *vs* chemoresistant, or metastatic *vs* non-metastatic disease. Studies comparing non-malignant bone *vs* OS tissue have not been earlier reported.

In this study, we compared the transcriptomes of chemo-naive OS biopsies, collected at the time of diagnosis, with samples of non-malignant bone. Statistical analysis of the expression profiles shows that osteosarcomas are characterised by an early deregulation of genes involved in drug resistance, tumour progression, antigen presentation, and osteoclastogenesis. Furthermore, in biopsies from patients who developed metastatic disease, these changes were significantly more pronounced. These data suggest that patient prognosis is determined early in tumour development and that enhancing antigen presentation or osteoclastogenesis may be of clinical value in treating OS.

MATERIALS AND METHODS

Patient samples

Patients presented to the Oncology Clinic at the Princess Alexandra or at The Wesley Hospitals (Brisbane, Queensland, Australia). Tumour biopsies were collected at the time of initial

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Table I Clinical information for the osteosarcoma patients used in the study

Tumour ID	Age (years)	Gender	Site of tumour	Tumour necrosis (%)	Response
G3	15	М	Proximal tibia	100	R
S4	29	F	Sacrum	<5	Ν
S5	27	F	Proximal humerus	<50	Ν
S7	39	Μ	Pelvis	<50	Ν
M8	24	F	Distal femur	25	Ν
L9	17	Μ	Tibia	75	Ν
W10	14	Μ	Distal femur	75	Ν
AI3	7	F	Proximal tibia	80	Ν
MI4	67	Μ	Pelvis	75	Ν
S15	76	F	Tibia	<90	Ν
W16	15	F	Proximal Humerus	<90	Ν
017	12	Μ	Distal femur	75	Ν
MI8	18	Μ	8th rib	98	R
K19	17	М	Proximal tibia	50	Ν
E20	13	F	Femur	95	R
M21	15	М	Distal femur	95	R
W22	15	F	Femur	80	Ν
G23	18	Μ	Distal femur	80	Ν
F2BR	18	Μ	Proximal tibia	80	Ν
A3BR	19	F	Distal femur	80	Ν
V4BR	16	Μ	Calcaneum	<5	Ν
M7BR	4	F	Distal femur	>92	R
Tl ^a	37	F	Proximal tibia	U	U

Response to chemotherapy: R = good response; N = poor response; U = unknown. ^aThis patient was used in the comparison between osteosarcoma and non-malignant bone, but not in the chemotherapy response study.

diagnosis, before preoperative chemotherapy, with informed consent from patients/guardians and with approval from the relevant institutional Research Ethics Committees. Twentythree biopsies were available and subjected to gene expression profiling analysis. Clinical data detailing response to chemotherapy was available for 22 out of 23 patients (Table 1). Patients were classified as good responders (R) if the tumours had \geq 90% tumour necrosis, or poor responders (N) if the tumours had <90% necrosis in response to preoperative chemotherapy (doxorubicin, 25 mg m^{-2} and cisplatin, 100 mg m^{-2}) as determined by histologic examination at the time of definitive surgery (Salzer-Kuntschik et al, 1983). Non-malignant bone was collected with consent from five patients presenting for hip or knee replacement surgery.

Microarray and data analysis

Extraction of RNA from cells and tumours was performed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Each OS patient sample was analysed in duplicate using dye swapping. Five nonmalignant bone samples were analysed individually. Labelled reference RNA and labelled tumour RNA were combined before hybridisation to Agilent Whole Human Genome Oligo Microarrays (Agilent Technologies, Santa Clara, CA, USA). Arrays were scanned on an Agilent DNA Microarray Scanner G2505B (Agilent Technologies). The microarray data discussed in this work have been deposited in NCBI's Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO series accession number GSE19276. Data extraction was performed using ImaGene version 6.1 (BioDiscovery Inc, El Segundo, CA, USA). Statistical analysis of the data was performed with GeneSpring GX software versions 7.2 and 10.0.1 (Agilent Technologies). Details of the analysis are described in Supplementary Material.

PCR analysis

For validation, 11 genes were selected at random from the 20 most highly differentially expressed molecules between non-malignant bone and OS. For PCR, $2 \mu g$ of non-malignant bone or OS tumour biopsy RNA was reverse transcribed with BioScript (Bioline, Sydney, Australia); PCR was performed using Taq DNA polymerase with ThermoPol II buffer (New England Biolabs, Ipswich, MA, USA) at an annealing temperature of 53 – 55°C for 30 cycles on a ThermoHybaid PxE0.2 (Thermo Scientific, Waltham, MA, USA). Primers were as follows: ID1 (forward 5'-CGGATCTGAGGGA GAACAAG-3' and reverse 5'-CTGAGAAGCACCAAACGTGA-3'), PRDX4 (forward 5'-GAGGACTTGGGCCAATAAGG-3' and reverse 5'-TTCACTACCAGGTTTCCAGC-3'), TPM2 (forward 5'-CGAGAG TAAATGTGGGGACC-3' and reverse 5'-TAAAGGATGAAGCCA GTGCC-3'), MT1E (forward 5'-TGCTTGTTCGTCTCACTGG-3' and reverse 5'-AAAGAAATGCAGCAAATGGC-3'), FKBP9 (forward 5'-TACCTGAAAACTGTGAGCGG-3' and reverse 5'-GTTCATCT GGTTTGGCTTCC-3'), S100A13 (forward 5'-ACCTTATGACCTGT CAGCCC-3' and reverse 5'-CCGAGTCCTGATTCACATCC-3'), S100A8 (forward 5'-TGGGCATCATGTTGACCGAGCTG-3' and reverse 5'-GCCACGCCCATCTTTATCACCAGA-3'), CTSG (forward 5'-CG CATCTTCGGTTCCTACG-3' and reverse 5'-GCTTCTCATTGTT GTCCTTATCC-3'), VWA5B2 (forward 5'-TACTCGGGAGCTAC TCTTCC-3' and reverse 5'-CATATGGCTGTGTCAGAGGG-3'), AZU1 (forward 5'-AGCATCAGGTCGTTCAGGTT-3' and reverse 5'-CAGAATCAAGGCAGGCACTTC-3'), PFC (forward 5'-GCTCT GTCACCTGCTCCAA-3' and reverse 5'-GCGGCTTCGTGTCTC CTTA-3').

RESULTS

Gene expression profiling of OS vs non-malignant bone

We compared gene expression in 23 OS biopsies and 5 nonmalignant bone samples. Our analysis yielded a suite of 305 differentially expressed genes (two-fold or greater, P < 0.05) between OS and non-malignant bone, of which 206 were annotated (Supplementary Table 1). Table 2 lists the 10 most highly up and 10 most highly downregulated genes, 11 of which were selected at random and their differential expression confirmed by PCR in two non-malignant bone samples and in five randomly selected OS tumour biopsies (Figure 1). Of the 36 upregulated genes, 47% were associated with cellular growth and proliferation (e.g. ID1, ANXA2, BTG3, MT2A, ITGB1, NDUFAF2). The most wellrepresented family of genes within this group was the metallothionein family, linked to intrinsic and acquired drug resistance (Cherian *et al*, 2003). Seven members of this family, MT1E, MT1H, MT1X, MT2A, MT1B, MT1G, and MT1L, were upregulated in our OS samples, and three were among the 10 most highly upregulated genes (Table 2). The inhibitor of DNA binding 1, ID1 (+4.07, P=0.003), peroxiredoxin 4, PRDX4 (+3.63, P=0.007), S100 calcium-binding protein A13, S100A13 (+2.66, P = 0.009), annexin 2, ANXA2 (+2.62, P = 0.003), and destrin, DSTN (+2.50, P = 0.001), earlier reported as positive regulators of angiogenesis, tumour progression and invasion (Fong et al, 2003; Landriscina et al, 2006; Estornes et al, 2007; Lee et al, 2008; Mussunoor and Murray, 2008; Iwatsuki et al, 2009) were all induced in OS samples (Table 2).

Analysis of the 170 downregulated genes found a large number associated with the inflammatory (26%) and cell-mediated (31%) immune response, as well as with antigen presentation (24%). Ingenuity pathway analysis (IPA) (Ingenuity Systems, Mountain View, California, USA) identified the antigen-presentation pathway as downregulated in OS with HLA-C, HLA-DOA, HLA-DPB1, HLA-DPA1, and HLA-E all expressed 2.17- to 3.45-fold lower in the lesions than in non-malignant bone (P = 0.002 - 0.04). However, the most highly differentially expressed gene was

Probe ID Symbol		Description and accession number	Fold change	P-value	
A_23_P252306	IDI	Inhibitor of DNA binding 1, dominant-negative	4.07	0.00276	
		helix-loop-helix protein [NM_002165]			
A_23_P114232	PRDX4	Peroxiredoxin 4 [NM_006406]	3.63	0.00664	
A_23_P216501	TPM2	Tropomyosin 2 (β) [NM_213674]	3.30	0.00237	
A_23_P206724	MTIE	Metallothionein IE [NM_175617]	3.11	0.00661	
A_23_P334709	FKBP9	FK506-binding protein 9, 63 kDa [NM_007270]	3.08	0.0111	
A_23_P163782	MTIH	Metallothionein IH [NM_005951]	3.02	0.0169	
A_24_P125096	MTIX	Metallothionein IX [NM_005952]	2.72	0.0316	
A_23_P372874	S100A13	SI00 calcium-binding protein AI3 [NM_005979]	2.66	0.00955	
A_32_P94798	ANXA2	Annexin A2 [NM_001002857]	2.62	0.00359	
A_23_P408095	DSTN	Destrin (actin depolymerising factor) [NM_001011546]	2.50	0.00140	
A_23_P434809	S100A8	SI00 calcium-binding protein A8 (calgranulin A) [NM_002964]	-100.00	0.00549	
A_23_P37856	HBAI	Homo sapiens haemoglobin, α Ι (HBAI), mRNA [NM_000558]	-50.00	4.26E-10	
A_23_P140384	CTSG	Cathepsin G [NM_001911]	-16.67	0.0222	
A_23_P80867	VWA5B2	von Willebrand factor A domain containing 5B2 [AL834499]	-11.11	0.0222	
A_23_PI53741	AZUI	Azurocidin I (cationic antimicrobial protein 37) [NM_001700]	-6.67	0.0485	
A_23_P22444	CFP	Properdin P factor, complement [NM_002621]	-6.25	0.0496	
A_23_P208866	GMFG	Glia maturation factor, γ [NM_004877]	-5.88	0.00748	
A 24 P207195	IRX3	Iroquois homeobox protein 3 [NM 024336]	-5.88	0.00263	
A 23 P403886	GLYAT	Glycine-N-acyltransferase [NM 005838]	-5.56	0.00391	
A_23_P156708	TNXB	Tenascin XB [NM_019105]	-5.56	0.00351	

Top 10 upregulated and 10 downregulated genes between osteosarcoma biopsies and non-malignant bone samples.



Figure I Validation of highly differentially expressed genes in osteosarcoma vs non-malignant bone. Eleven genes were selected at random from Table I and validated by PCR in two non-malignant bone samples (lanes I and 2), and five randomly selected osteosarcoma patients (lanes 3-7). The results are shown in groups of genes upregulated and downregulated in osteosarcoma compared with non-malignant bone.

the S100 calcium-binding protein A8 (S100A8), a marker of a number of inflammatory conditions (Zreiqat et al, 2007), which was downregulated 100-fold in OS (P = 0.005) (Table 2). Also downregulated were cathepsin G (CTSG; -16.67, P = 0.02) and azurocidin 1 (AZU1; -6.67, P = 0.05), which regulate monocyte/ macrophage function and chemotaxis in inflammatory conditions (Pereira, 1995; Miyata et al, 2007).

The OS biopsies also revealed a transcriptomic signature characteristic of reduced osteoclastogenesis. ID1 is an inhibitor of osteoclast differentiation (Lee et al, 2006) and was induced fourfold in OS biopsies. Similarly, there was a 100-fold downregulation of S100A8, which is highly expressed in osteoclasts (Zreiqat et al, 2007), and significantly lower expression of another 13 genes associated with negative regulation of osteoclast differentiation/function, or indicating diminished osteoclast presence or activity. These genes included von Willebrand factor A domain, 5B2 (VWA5B2; -11.11, P = 0.02), FGR, a member of the Src family of protein tyrosine kinases (-5.56, P = 0.02), TYRO protein kinase-binding protein (TYROBP; -5.26, P = 0.04), the Rac small GTPase RAC2 (-4.76, P = 0.01), RelA/p65 (-4.55, P = 0.005), MYC (-3.70, $P = 2.77 \times 10^{-16}$), signal regulatory protein α (SIRP α /SIRPA; -2.94, P=0.006), tartrate-resistant acid phosphatase (ACP5/TRAP, -2.86, P=0.005), BCL2 (-2.5-fold, P = 0.005), high-mobility group box 1 (HMGB1; -2.33, P = 0.002), V-ATPase (ATP6V0D1; -2.27, P = 0.04), leukotriene B4 receptor (LTB4R; -2.22, P=0.03), and gelsolin (GSN, -2.08, P=0.04). To see whether the reduction in ACP5/TRAP gene expression correlated with a decrease in the number of osteoclasts in OS, we performed immunohistochemistry on FFPE sections of OS biopsies and of non-malignant bone with a monoclonal antibody to ACP5/TRAP. We found a 2.5-fold decrease in the number of osteoclasts in OS biopsies compared with non-malignant bone, which correlated with the observed 2.3-fold decrease in ACP5/ TRAP gene expression (Figure 2A). Furthermore, the decrease in ACP5/TRAP expression was significantly more marked in the biopsies of patients, which showed a poor response to chemotherapy treatment than in those who exhibited a good response (Figure 2B), suggesting that a reduction in osteoclastogenesis is not only associated with OS in general, but also with chemoresistance.

Gene expression profiling of good responders vs poor responders

Osteosarcomas are inherently drug-resistant tumours (Chou and Gorlick, 2006), and, therefore, the most commonly used predictor of disease outcome is a patient's initial response to chemotherapy. Unfortunately, this response cannot be assessed at the time of presentation. To specifically search for genes that could be predictive of chemotherapeutic response and drug resistance at the time of diagnosis, patients were divided into good (n = 5) and poor (n = 17) responders using the criteria already described. A set of 123 genes was found to be significantly differentially expressed (P < 0.05) between the two groups, of which 61 were annotated (Table 3). Most of these genes (94%) were upregulated in the good responders and were associated with cellular development, growth, and proliferation, suggesting that good responders may have tumours that are more proliferative and may, therefore, be more sensitive to the effects of chemotherapy. Among the most highly



Figure 2 Impaired osteoclastogenesis in osteosarcoma correlates with chemoresistance. (A) Osteoclast number is expressed as the average number of osteoclasts per \times 20 field in immunohistochemically stained FFPE sections from osteosarcoma biopsies (OS) and non-malignant bone (NB). The ACP5 expression as measured by microarray analysis and expressed as normalised intensity. ***P < 0.0001, *P = 0.0491. Bars: mean + s.e.m. (B) ACP5 expression in good and poor responders, as measured by microarray analysis and expressed as normalised intensity. **P < 0.0491. Bars: mean + s.e.m.

upregulated genes in good responders was thymosin β 10 (TSMB10, + 5.34-fold, *P*=0.017), which had been identified within the suite of differentially expressed genes between good and poor responders in an earlier study of OS tumour biopsies (Ochi *et al*, 2004).

To test the suitability of this set of 123 genes to separate between good and poor responders, we performed unsupervised hierarchical clustering of the data. With the exception of one patient (M18), the gene set was able to clearly separate 21 out of 22 (>95%) OS patients on the basis of their response to chemotherapy (Figure 3). We examined the possibility that some of these genes could serve as individual predictors of chemotherapeutic response. We selected individual genes from the list based on their \geq threefold expression (Table 3), and looked at their levels of expression in individual patients. Of the selected genes, only TMSB10, SPP1, CTSB, TYROBP/DAP12, and IFI30 showed significant (P<0.05) differential expression between patients in the two groups, with IFI30 (P=0.0005) showing the most significant difference (Figure 4).

One of the major obstacles to effective treatment of OS patients is intrinsic or acquired resistance to the cytotoxic effects of anticancer agents. The mechanism dictating this resistance in OS is still unknown, but may involve a number of gene families, which mediate detoxification, increased efflux from the cell, and increased DNA repair (Chou and Gorlick, 2006). Therefore, understanding the mechanism of drug resistance and identifying genes that are involved may lead to new therapies that could improve survival. The cytochrome P450 family of enzymes functions in the detoxification of anticancer drugs (Simpson, 1997). As our signature found CYP4X1 differentially expressed between good and poor responders, we looked at the mRNA expression levels in each patient of CYP4X1 and other cytochrome P450 family members (Figure 5F). Of the 50 or more P450 genes present on the array, only 19 had detectable expression levels in our tumour biopsies, and of these, only CYP4X1 had significant differential expression associated with chemotherapeutic response. However, given that CYP4X1 is an orphan P450 protein with no assigned biological function (Stark et al, 2008), and that it was downregulated in good responders, its function in OS drug response remains unclear.

Other enzyme families responsible for resistance to many chemotherapeutic agents include the glutathione-S-transferases (GSTs) (Ekhart *et al*, 2009) and the ATP-binding cassette (ABC) transporters (Sharom, 2008). We, therefore, compared the expression of GST and ABC family members between good and poor responders. Only five GSTs were expressed in our samples, but none significantly (Figure 5A). Similarly, only one ABC transporter, ABCG2, was found expressed in our samples, but its levels were not significantly different between the two groups (Figure 5C). Moreover, we found no significant difference in the expression of other genes involved in DNA damage response, drug metabolism, apoptosis, or survival (Figures 5B, D and E). Taken together, our data indicate that genes classically associated with multi-drug resistance do not correlate with chemotherapeutic response in OS.

DISCUSSION

Despite intensive multi-agent chemotherapy, OS remains an aggressive, highly metastatic, and relatively drug-resistant tumour with poor long-term survival rates (Chou and Gorlick, 2006). The mechanisms behind metastasis and chemoresistance in OS are not well understood, but are likely to be due to the innate biology of metastatic and chemoresistant lesions (Gorlick and Meyers, 2003). Therefore, understanding the basic tumour biology is central to understanding OS pathogenesis and chemoresistance. In this study, we used chemo-naive OS biopsies and, for the first time, compared their transcriptomes to those of non-malignant bone. We identified a unique gene signature showing increased expression of genes associated with tumour progression and drug resistance, and decreased expression of genes associated with antigen presentation and osteoclastogenesis in all OS lesions. In addition, tumours that were chemoresistant were characterised by more pronounced inhibition of osteoclastogenesis markers and antigen-presenting activity than tumours that were chemosensitive. We noted no significant difference in the expression of genes classically associated with drug resistance between chemosensitive and chemoresistant tumours.

This study identified ID1 as a potentially important molecule in the regulation of many of the characteristics of OS. Increased expression of ID1 in the OS biopsies has been shown to be involved in the proliferation, survival, angiogenesis, metastasis (Ling et al, 2006), and formation of a permissive metastatic niche in other cancer types (Lyden et al, 1999), and may be similarly involved in OS. Recently, ID1 has been identified as a novel inhibitor of PTEN and p53, leading to AKT-dependent activation of the canonical Wnt-signalling pathway (Lee et al, 2009). The Wnt-signalling pathway has earlier been shown to be important to the regulation of OS progression (Hoang et al, 2004; Geryk-Hall and Hughes, 2009; Kansara et al, 2009). Thus, there is evidence that the overexpression of ID1 in OS may be causally involved in the growth, survival, and metastatic behaviour of OS. If true, then targeting of the Akt and Wnt pathways could be of value in OS (Geryk-Hall and Hughes, 2009).

Mintz et al (2005) earlier reported that genes involved in osteoclast differentiation and function in OS were mostly associated with a poor chemotherapeutic response. We now report that osteoclast numbers are decreased in all OS lesions. Thus, the loss of osteoclasts in OS may be involved in OS metastasis, although the mechanism by which OS induce osteoclast loss is unknown. An earlier transcriptomic study in seven OS biopsies (Patino-Garcia et al, 2009) showed up-regulation of EBF2, a known

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Table 3 Good response vs poor response

NH_0103 The Bit D Thread (I 0 Component of the second propher (I - Component), functional of the second propher (I - Compo	Genbank	Synonym	Common name	Fold change	P-value
NPL_00052 SPI1 Societed phaspication 1 (obseption), how subported in (obseption), how subported in the subported in the subport i	NM 021103	TMSB10	Thymosin, β 10	5.34	0.0171
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NM_06332 IFI30 Interferon, p-reducible protein 30 4.35 00224 NM_020251 HR50 Migor Intercomptability complex class I, 6 3.36 00244 NM_020511 HR51 Migor Intercomptability complex class I, 6 3.36 00244 NM_020511 HR51 Cathegin B and II (Nucle-Teglens syndrame) 3.46 00245 NM_020502 FIHI Formin Intercomptability complex class I, A 3.17 00251 NM_020512 FIHI Formin Intercomptability complex class I, A 3.17 00251 NM_00012 RFIF9 Riboarnal protein 151 3.16 00231 NM_00012 RFIF9 Riboarnal protein 152 3.16 00231 NM_00012 RFIF9 Riboarnal protein 152 3.11 00471 NM_00012 RFIF9 Riboarnal protein 152 3.11 00471 NM_00017 HLA-C Migar hitoacomptability complex clast I, C 2.59 00401 NM_00017 HLA-C Migar hitoacomptability complex clast I, C 2.59 00401 NM_000117 HLA-C <td></td> <td></td> <td>early T-lymphocyte activation 1)</td> <td></td> <td></td>			early T-lymphocyte activation 1)		
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NML_00511 HI-A-B Major hatocompability complex, clas I, B 3.35 0.0487 NML_00703 STK11 Structure-Topics optimum 3.49 0.0233 NML_00703 CTS Cahoson B 3.49 0.0233 NML_00703 LTS Cahoson B 3.49 0.0233 NML_00703 LTH Lemma have polypother I 3.16 0.0247 NML_002116 HLA-A Major have polypother ompice, class I, A 3.17 0.0237 NML_00212 RTRD P Ribosom protein structure protein 3.16 0.0487 NML_00212 RR-1P Ribosom protein lings, 60 3.16 0.0487 NML_002117 HLA-C Major hatocompability complex, class I, C 2.99 0.099 NML_002117 HLA-C Major hatocompability complex, class I, C 2.99 0.099 NML_002117 HLA-C Major hatocompability complex, class I, C 2.99 0.099 NML_002117 HLA-C Major hatocompability complex, class I, C 2.99 0.099 NML_002020 HHLI-C Lonany poto	NM_002952	RPS2	Ribosomal protein S2	3.70	0.0487
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NPL (4/740) L.198 Campan B 4.48 00353 NPL (356) LITTRE, bit intrickin IT resigner C 332 00415 NPL (0550) CFL Colin I (con-marke) 327 00371 NPL (0533) TTROP Resonal protein (since binding protein) 316 00435 NPL (0533) TTROP Resonal protein (since binding protein) 316 00437 NPL (05332) TRROP Resonal protein (since binding protein) 314 00497 NPL (00012) RPLPQ Resonal protein (since binding protein) 314 00497 NPL (00012) RPLPQ Resonal protein (since binding protein (since binding protein) 314 00497 NPL (0014) REFIG Evaluation transition strate, IC 299 00431 NPL (0014) REFIG Evaluation transition strate, IC 293 00433 NPL (00102) RPLP Resonal protein (since transmotione protein 2 (1402) 245 00392 NPL (00102) RPLP Resonal protein (since transmotione protein 2 (1402) 245 00444 NPL (000297<	NM_000455	SIKII	Serine/threonine kinase 11 (Peutz–Jeghers syndrome)	3.49	0.0249
NPL 33740 LI //RC interfacient // receptor C 3.39 DUA13 NPL 020223 TTI lemits have polypoids 3.39 DUA13 NPL 02023 TTI lemits have polypoids 3.37 DUA33 NPL 03132 TYRCEP <	NM_147780	CISB	Cathepsin B	3.48	0.0355
NPL_02022 rtml rtml rtml Add Add <t< td=""><td>NM_153461</td><td>ILT/RC</td><td>Interleukin 17 receptor C</td><td>3.37</td><td>0.0415</td></t<>	NM_153461	ILT/RC	Interleukin 17 receptor C	3.37	0.0415
11 2000/fit 21 2000 11 11 11 21 2000 11 11 11 21 2000 11 11 11 21 21 21 11 11 11 21 21 21 21 11 11 11 11 21 21 21 21 21 21 21 21 21 21 21 21 21 22 21 22 23 0332 21 04 21 23 0332 21 04 21 23 03 04 11 24 04 11 24 04 11 11 04 04 04 04 04 04 04 04 04 04 04 04 04 04 04 11 11 04 04 04 04 04 04 04 04 04 04 04	NM_005507		Ceflin I. (non musclo)	3.3Z 2.20	0.0355
International State 1.1 0.0047 MPLD0102 PR519 Record protein Signation in the interim sis in the interemation signation in the interim sis in the interim			Maian histo assess tibility assesses (alass I. A	3.27	0.0251
NuCl.201332 1 No. Jp. 11 No. Jp. 11 No. Jp. 2014 3.16 0.003 NuCl.201332 RR.129 Ribition in the provide in the structure integer protein (17.8 k/s. hepan/epanan sulphate 3.14 0.0047 NuCl.201375 RR.129 Ribition in the provide integer protein (17.8 k/s. hepan/epanan sulphate 3.14 0.0047 NuCl.20137 HLA-C Major histocompability complex, class L.C 2.99 0.0041 NuCl.20147 HLA-C Major histocompability complex, class L.C 2.93 0.0043 NuCl.201404 EFI-IG Europatility complex, class L.C 2.93 0.0041 NuCl.201404 RFI-ID Representation of the structure integer protein for the structure integer protein integ	NM_002222		Major histocompatibility complex, class I, A	3.17	0.0379
NML003972 RPL39 Reboomd potent 1/2 mt R 2.14 0025 NML000972 RPL39 Reboomd potent 1/2 kak hepsin/egara subject 3.11 0.0047 NML00177 HLA-C Major Instance and the potent 1/2 kak hepsin/egara subject 3.11 0.0047 NML00104 EEFIC Evaluation of the potent 1/2 kak hepsin/egara subject 2.93 0.0041 NML00104 EEFIC Evaluation transmitter on compation factor 1 7 2.93 0.0043 NML00104 RFS10 Rebosomal potent Reg, PI 2.81 0.0397 NML00102 RFF14 Compatibility complex, classificatily controlled 1 2.66 0.0352 NML00102 RFS16 Rebosomal potent L15 2.66 0.0457 NML00102 RFS16 Rebosomal potent L15 2.66 0.0458 NML001020 RFS16 Rebosomal potent L21 2.56 0.0448 NML001037 RFL18 Rebosomal potent L21 2.76 0.0449 NML00104 RFS16 Rebosomal potent L21 2.77 0.0452 NML00037 RFL18	NM 001022		Pilocomol protein STA	216	0.0467
NM_00099 PR129 Nitbosomal proton 1/2 s.t.a. hepan/epanan subpate 1.1 00049 NM_000117 HLA-C Migh ristocompatibility complex, clast I, C 299 00401 NM_001040 EEFIG Eukaryote translation clongation factor 1 y 233 00443 NM_001041 RFSI Bularyote translation clongation factor 1 y 283 00171 NM_00103 RPLP1 Rubosomal proton 10.7 283 00171 NM_00103 RPLP1 Rubosomal proton 10.7 283 00171 NM_001041 RFSI Rubosomal proton 11.5 266 00325 RC31631 CFLP1 Cafilin pseudogene 1 266 00439 NM_00042 RPL15 Rubosomal proton 11.5 260 00437 NM_000592 RPL1 Rubosomal proton 11.5 260 00435 NM_000592 RPL18 Rubosomal proton 11.5 260 00444 NM_000592 RPL18 Rubosomal proton 11.5 247 00332 NM_000592 RPL19 Rubosomal proton 11.5 247 00434	NM 053275		Ribosomal protein Jarga PO	3.10	0.023
Not 0.002.02 Initial control interacting provider, Helly Initial regularization of the standard provider of the s	NM 000992	RPI 29	Ribosomal protein 129 alka henarin/enaran sulphate	3.14	0.0497
NPL 0021 / I HLA-C Major histocompatibility complex, diss L.C. 2.99 0.04401 NR 002205 FTHL12 Fortin, heavy ophyperide.life 12 295 0.0332 NM 001014 RFI0 Elakarycic translation elongiation factor 1 y 293 0.0143 NM 00103 RPLP1 Ribosomal protein.large, P1 283 0.0171 NM 00103 RPLP1 Ribosomal protein.large, P1 266 0.0365 BC331631 CFLP1 Coffin pseudogene 1 266 0.0365 RVM_000432 RFL1 Ribosomal protein L15 260 0.0497 NM_000792 RPL16 Ribosomal protein S16 258 0.0355 NM_000797 RPL18 Ribosomal protein L18 250 0.0455 NM_00102 RFS19 Ribosomal protein S191 247 0.0337 NM_00102 RFS19 Ribosomal protein S191 247 0.0339 NM_00102 RFS19 Ribosomal protein S191 247 0.0349 NM_00107 FAU FAU FAU Elabosonal protein S191 <td>111_000772</td> <td></td> <td>interacting protein (HIP)</td> <td>5.11</td> <td>0.0177</td>	111_000772		interacting protein (HIP)	5.11	0.0177
NR_020205 PTHL12 Ferritin, heavy polypeptide-like 12 Construction 295 002322 NRL_001044 EFS10 Ribosomal protein, Ingr. PI 283 00171 NRL_00103 RPLPI Ribosomal protein, Ingr. PI 281 00355 NRL_00103 RPLPI Tumour protein, Ingr. PI 286 00355 NRL_0023255 TPT1 Tumour protein, Ingr. PI 266 000325 NRL_002348 RPL15 Ribosomal protein Ingr. PI 266 000457 NRL_002048 RPL15 Ribosomal protein Ingr. PI 256 00455 NRL_001020 RPS16 Ribosomal protein IS 251 00444 NRL_001020 RPS16 Ribosomal protein IS 251 00445 NRL_001027 RPS19 Ribosomal protein IS 251 00445 NRL_001027 RPS19 Ribosomal protein IS 245 00451 NRL_001027 RPS19 Ribosomal protein IS 245 00451 NRL_001027 FS19 Ribosomal protein IS 245 00454 <td>NM 002117</td> <td>HIA-C</td> <td>Major histocompatibility complex class L C</td> <td>299</td> <td>0.0401</td>	NM 002117	HIA-C	Major histocompatibility complex class L C	299	0.0401
NPL_001404 EFI G Extransition elongrin factor 1 y 2.93 0.0443 NPL_00103 RFLP1 Risocomal protein, large, P1 2.81 0.0377 NPL_001035 TFT1 Tumour protein, transitionally controlled 1 2.66 0.0355 R0111631 CFLP1 Cofiin pseudogene 1 2.66 0.0357 NPL_00435 IFTMP1 Inferform induced transmembrane protein 2 (1-8D) 2.61 0.0497 NPL_001020 RF16 Riboornal protein 1.15 2.56 0.0497 NPL_002032 COAS2 Crydophilin LC 2.51 0.0495 NPL_002032 RF21 Riboornal protein 516 2.50 0.0495 NPL_00102 RF51 Riboornal protein 1.81 2.47 0.0397 NPL_00102 RF51 Riboornal protein 519 2.47 0.0493 NPL_00103 RF21 Tamour protein, transitionally controlled 1 2.47 0.0493 NPL_001097 FAU Firled-Bibles-Relly murine sarcma virus (BR-MSV) 2.45 0.0047 NPL_001097 FAU FAU <t< td=""><td>NR 002205</td><td>FTHL12</td><td>Eerritin, heavy polypentide-like 12</td><td>2.95</td><td>0.0392</td></t<>	NR 002205	FTHL12	Eerritin, heavy polypentide-like 12	2.95	0.0392
NML 001014 RPS10 Ribosomal protein S10 2.9.3 0.0171 NML 00103 RPLP1 Ribosomal protein, large, Pl 2.81 0.0335 NML 0023925 TPT1 Tumour protein, large, Pl 2.66 0.0325 NML 002435 IFTN4 Rubosomal protein L15 2.66 0.0425 NML 002448 RPL15 Ribosomal protein L15 2.56 0.0459 NML 000700 RPS16 Ribosomal protein L12 2.56 0.0459 NML 000797 RPL18 Ribosomal protein L18 2.50 0.0444 NML 000797 RPL18 Ribosomal protein L18 2.50 0.0459 NML 00122 RPS19 Ribosomal protein S19 2.47 0.0439 NML 00123 RPS17 Rubosomal protein N19 2.45 0.0214 NML 00124 RPS16 Ribosomal protein S19 2.45 0.0437 NML 00125 TPT1 Tumour protein transelitonally controlled 1 2.47 0.0439 NML 00106 RPS1A Ribosomal protein S19 2.45 0.0437	NM 001404	FFFIG	Eukaryotic translation elongation factor L v	2.73	0.0443
NH_001003 PRP1 Rhosomal protein, large, Pl 281 00397 NH_00225 PT1 Tumour protein transditionally controlled 1 265 00316 NM_00435 IFTM2 Interferon induced transmembrane protein 2 (1-8D) 261 00497 NM_004784 RPL15 Rhosomal protein L15 258 00435 NM_00100 RP516 Rhosomal protein S16 258 00439 NM_000979 RP1.21 Rhosomal protein S16 258 00439 NM_00102 RP518 Rhosomal protein S19 247 00439 NM_00102 RP518 Rhosomal protein S19 247 00439 NM_0010979 RPL18 Rhosomal protein S19 247 00439 S0204271 FANCC Fanconi anaremi, complementation group C 245 00431 NM_00106 RP53A Rbosomal protein S16 245 00431 NM_00106 RP53A Rbosomal protein S16 245 00431 NM_00106 RP53A Rbosomal protein S16 240 00444	NM 001014	RPS10	Bibosomal protein SIO	2.73	00171
NM_003295 TPT I Tumour protein transitionally controlled I 2.66 00335 NM_002435 IFITM2 Interferon induced transmembrane protein 2 (1-8D) 2.61 00497 NM_002435 IFITM2 Interferon induced transmembrane protein 2 (1-8D) 2.60 0.0497 NM_00200 RPLI6 Ribosomal protein 121 2.56 0.0497 NM_00202 RPL3 Ribosomal protein 121 2.51 0.0448 NM_00202 RPS19 Ribosomal protein 121 2.47 0.0452 NM_00202 RPS19 Ribosomal protein 141 2.47 0.0452 NM_00202 RPS19 Ribosomal protein scattorin averus (RBA.McSV) 2.45 0.0214 NM_00106 RPS3A Ribosomal protein S3A 2.45 0.0497 NM_00106 RPS3A Ribosomal protein S3A 2.45 0.0497 NM_00106 RPS3A Ribosomal protein S1A 2.32 0.0497 NM_00107 RP3A Ribosomal protein S1A 2.32 0.0497 NM_00106 RPS3A Ribosomal protein S1A	NM 001003	RPI PI	Ribosomal protein large Pl	2.85	0.0397
BC03131 CFLP1 Collin pseudogene 1 265 00232 NML00435 FITM2 Interferon induced transmembrane protein 2 (1-8D) 261 00499 NML002948 RPL15 Ribosomal protein 516 260 00497 NML00292 RPL21 Ribosomal protein 118 256 00499 NML00292 RPL21 Ribosomal protein 118 250 00455 NML00292 RPL21 Ribosomal protein 118 250 00455 NML001022 RP519 Ribosomal protein 118 247 00399 NML00122 RP519 Ribosomal protein 118 247 00439 NML00129 FP11 Tumour protein translationally controlled 1 247 00439 NML00129 FAICC Fanconi namemia, complementation group C 245 00431 NML00129 FAI Fanconi namemia, complementation group C 245 00497 NML00129 FAICC Fanconi namemia, complementation group C 245 00497 NML00120 RP3A Ribosomal protein SA 245	NM 003295	TPTI	Tumour protein, translationally controlled I	2.61	0.0365
$\begin{split} & \begin{tabular}{ c c c c c c c c c c c c c c c c c c c$	BC031631	CELPI	Cofilin proteini, alabilationalifi controlled i	2.65	0.0232
ML 002948 RPL IS Ribosomal protein L15 2.60 0.0979 NML 001020 RS16 Ribosomal protein S16 2.58 0.0355 NML 000802 RPL21 Ribosomal protein L11 2.56 0.0444 NML 000802 RPL21 Ribosomal protein L18 2.50 0.0444 NML 001022 RRS19 Ribosomal protein L18 2.50 0.0445 NML 001022 RRS19 Ribosomal protein L18 2.47 0.0393 BC034271 FANCC Fancori anaemia, complementation group C 2.45 0.0417 NML 00106 RPS3A Ribosomal protein S3A 2.45 0.0497 NML 00106 RPS3A Ribosomal protein S2A 2.45 0.0497 NML 00106 RPS3A Ribosomal protein S2A 2.45 0.0497 NML 00248 NDLP44 NADH 484/progenase (ukipainone) 1.a subcomplex.4, 9kDa 2.32 0.0434 NML 00248 NDLP44 NADH 484/progenase (ukipainone) 1.a subcomplex.4, 9kDa 2.32 0.0434 NML 002107 HSF3A Ribosomal protein S15a	NM 006435	IFITM2	Interferon induced transmembrane protein 2 (1-8D)	2.65	0.0489
NML00100 RP516 Ribosomal protein S16 258 0.0355 NML000982 RPL21 Ribosomal protein L21 256 0.0489 NML000979 RPL18 Ribosomal protein L18 250 0.0454 NML00102 RP519 Ribosomal protein S19 2.47 0.0392 NML001022 RP519 Ribosomal protein S19 2.47 0.0392 NML0010977 FAU Fincein aneximic, complementation group C 2.45 0.0214 NML00106 RPS3A Ribosomal protein S3A 2.45 0.0497 NML00106 RPS3A Ribosomal protein S3A 2.45 0.0497 NML00106 RPS3A Ribosomal protein S3A 2.45 0.0497 NML00106 RPS3A Ribosomal protein S1A 2.45 0.0492 NML002060 RPL2A Ribosomal protein S1A 2.45 0.0492 NML002060 RPS3A Ribosomal protein S1A 2.30 0.0332 NML002010 RPS1A Ribosomal protein S1A 2.30 0.04932 NML0020200	NM 002948	RPL15	Ribosomal protein 115	2.60	0.0497
NM_000982 RP21 Ribosomal protein L21 256 0.00489 NM_178230 COAS2 CyclophinLC 251 0.0444 NM_000979 RFL18 Ribosomal protein 118 250 0.0455 NM_001022 RP519 Ribosomal protein 519 247 0.0392 BC034271 FANCC Fancon anaemia, complementation group C 245 0.0214 NM_001997 FAU Finkel -Bdisk Really mutrics acroma vins (FBR-MuSV) 245 0.0493 NM_001066 RP53A Ribosomal protein 52A 245 0.0497 NM_001066 RP53A Ribosomal protein 52A 245 0.0497 NM_001066 RP53A Ribosomal protein 52A 245 0.0497 NM_001078 NL181464 TGBdBP Integrin β Honding protein 27a 239 0.0392 NM_0024807 NDUFA4 NADH dehydrogenase (ubquinone) 1 x subcomplex, 4, 9 kDa 232 0.0441 NM_0021018 RP142 Formin 2 230 0.0433 NM_002107 H3F3A H3 bistone, famiy 3A 230	NM 001020	RPS16	Ribosomal protein \$16	2.58	0.0355
$\begin{split} & NM _ TP330 & COAS2 & Cydophilin C & C \\ & N1 \\ NM _ D03779 & RPL18 & Rbosomal protein L18 & 2.50 & 0.0455 \\ N1 _ D032275 & TPT1 & Tunour protein L18 & 2.50 & 0.0455 \\ N1 _ D032275 & TPT1 & Tunour protein L18 & 2.50 & 0.0455 \\ N1 _ D032275 & TPT1 & Tunour protein, translationally controlled 1 & 2.47 & 0.0439 \\ D03275 & TPT1 & FAVCC & Fanconi aaremia, complementation group C & 2.45 & 0.0214 \\ N1 _ N010197 & FAV & FaVCC & Fanconi aaremia, complementation group C & 2.45 & 0.0497 \\ \mathsf{N1 _ D01066 & RP53A & Rbosomal protein S3A & 2.45 & 0.0497 \\ \mathsf{N1 _ D01066 & RP53A & Rbosomal protein S3A & 2.45 & 0.0497 \\ \mathsf{N1 _ D01066 & RP53A & Rbosomal protein S3A & 2.45 & 0.0498 \\ \mathsf{N1 _ O010166 & RP53A & Rbosomal protein S3A & 2.45 & 0.0498 \\ \mathsf{N1 _ O010166 & RP53A & Rbosomal protein S4A & 2.32 & 0.0434 \\ \mathsf{N1 _ O002489 & NDUFA4 & NADH dehydrogenase (ubiquinone) 1 x subcomplex, 4, 9 kDa & 2.32 & 0.0441 \\ \mathsf{N1 _ O002489 & NDUFA4 & NADH dehydrogenase (ubiquinone) 1 x subcomplex, 4, 9 kDa & 2.32 & 0.0493 \\ \mathsf{N1 _ O001019 & RP515A & Rbosomal protein 127a & 2.30 & 0.0437 \\ \mathsf{N1 _ O00601 & N1 & S100 \ calcium-binding protein A11 (calgizzarin) & 2.30 & 0.0437 \\ \mathsf{N1 _ O00601 & N1 & N1 & N1 \ Oncormal protein S15a & 2.32 & 0.0441 \\ \mathsf{N1 _ O00601 & N1 & N1 & N1 \ \mathsf{Oncormal protein C17a & 2.23 & 0.0171 \\ $ d subant & $ $	NM 000982	RPI 21	Ribosomal protein 121	2.50	0.0489
NM_000979 RPL I8 Rbosomal protein L18 2.50 0.0455 NM_001022 RPS19 Rbosomal protein S19 2.47 0.0392 NM_00125 TPT1 Tumour protein translationally controlled I 2.47 0.0392 BC034271 FANCC Fanconi anaemia, complementation group C 2.45 0.043 NM_001097 FAU Finkel -Bidsis-Relip munics acroma virus (FBK-MLSV) 2.45 0.049 NM_001066 RPS3A Ribosomal protein S3A 2.45 0.049 NM_181468 ITGB4PB Integrin <i>β</i> -Inding protein 2.40 0.0444 NM_00990 NPL/FA4 NADPI dehydrogenase (ubiquinone) 1 x subcomplex, 4, 9/bDa 2.32 0.0443 NM_001019 RPS1A Ribosomal protein L7a 2.30 0.0487 NM_002107 H3F3A H3 bitone, family 3A 2.32 0.0443 NM_005050 FMN2 Formin 2 2.30 0.0487 NM_0050509 NME4 Non-metastatic cells 4, protein expressed in 2.27 0.0355 NM_0050509 NME4 Non-meta	NM 178230	COAS2		2.50	0.0444
$\begin{split} & NM_001022 & RS19 & Rbsormal protein S19 & 247 & 0.0392 \\ NM_003325 & TPT1 & Tumour protein, translationally controlled 1 & 247 & 0.0392 \\ \mathsf{SO34271 & FANCC & fanconi anaemia, complementation group C & 245 & 0.014 \\ NM_001997 & FAU & Finkel-Bisks-Relly murne sarcoma virus (\mathsf{FBR-MSV) & 245 & 0.043 \\ & ubiquitousy experssed (fox derived); RFS30 & 245 & 0.0493 \\ UD=001006 & RFS3A & Rbsosmal protein S3A & 245 & 0.0499 \\ NM_011468 & ITGB4BP & Integrip \ \beta 4-binding portein (s (CD)70) & 245 & 0.0499 \\ NM_018468 & ITGB4BP & Integrip \ \beta 4-binding protein (s (CD)70) & 245 & 0.0439 \\ NM_002489 & NDUFA4 & NADH dehydrogenase (ubiquinone) 1 \ a subcomplex, 4, 9kDa & 232 & 0.0439 \\ \mathsf{AK078605 & FMN2 & Formin 2 & 232 & 0.0443 \\ NM_002107 & H3F3A & H3 bistone, family 3A & 230 & 0.0303 \\ \mathsf{NM_005013 & RP_10 & Rbsosmal protein S15a & 222 & 0.0443 \\ NM_002067 & NDA_1 & S10A= L1 + Integrip \ p 4 + protein expressed in & 227 & 0.0365 \\ NM_006013 & RP_10 & Rbsosmal protein 10 & 228 & 0.0487 \\ NM_006686 & ATPSE & ATPS + Arpsynthase, H+ transporting mitochondrial F1 complex, 2.23 & 0.0411 \\ \mathsf{MM_0024060 & NDA_1 & CSC16 & CD= SC22 & 0.0444 \\ NM_0028228 & ZN5887 & Zinc-finger protein SP7 & 2.22 & 0.0444 \\ NM_0028248 & ZN5887 & Zinc-finger protein SP7 & 2.22 & 0.0444 \\ NM_0028248 & ZN5887 & Zinc-finger protein SP7 & 2.14 & 0.0415 \\ MM_002040 & MCATI & Manosyl(c_i:13)_g/scprotein & A$ + bydratise (trifunctional protein) & 2.16 & 0.0465 \\ NM_0002406 & MCATI & Manosyl(c_i:13)_g/scprotein & A + bydratise (trifunctional protein), a subunit \\ MM_0002406 & MCATI & Manosyl(ce_i:13)_g/scprotein & 2.14 & 0.0415 \\ MM_0002406 & MCATI & Manosyl(ce_i:13)_g/scprotein & 2.14 & 0.0415 \\ MM_0002406 & MCATI & Manosyl(ce_i:13)_g/scprotein & 2.24 & 0.0377 \\ MM_015533 & SP24P1 & Atsin:24 \text{ bioding protein} 1 & 2	NM 000979	RPI 18	Bibosomal protein 118	2.51	0.0455
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	NM 001022	RPS19	Ribosomal protein \$19	2.30	0.0392
BC034271 FANCC Fancon anaemia complementation group C 2.45 0.0214 NM_001997 FAU Finkel-Bikis-Relily murine sarcoma vins (FBR-MuSV) 2.45 0.043 NM_001006 RF53A Ribosomal protein S3A 2.45 0.0447 NM_001006 RF53A Ribosomal protein S3A 2.45 0.0449 NM_00090 RI2TAA Ribosomal protein IC7a 2.39 0.0392 NM_002489 NDUFA4 NADH dehydrogenase (ubiquinone) I α subcomplex 4.9 kDa 2.32 0.0443 NM_002107 H3F3A Ribosomal protein S15a 2.30 0.0392 NM_002107 H3F3A H3 histone, famin 3A 2.30 0.0393 NM_006013 RPLI0 Ribosomal protein A11 (calgizzarin) 2.30 0.0318 NM_006886 ATPSE ATP synthase, H+ transporting, mitochondrial FI complex, 4.9 kDa 2.23 0.0447 NM_006886 ATPSE ATP synthase, H+ transporting, mitochondrial FI complex, 2.23 0.0141 NM_005886 ATPSE ATP synthase, H+ transporting, mitochondrial FI complex, 2.23 0.0447 NM_0	NM 003295	TPTI	Tumour protein translationally controlled I	2.17	0.0439
NM_001997 FAU Finkel-Biskis-Reilly murine sarcoma vins (FBR-MuSV) 2.45 0.043 NM_001006 RFS3A Rbosomal protein S3A 2.45 0.0497 AY353369 SIGLEC5 Sialic acid-binding (g-like lectin S (CD 170) 2.45 0.0497 AY353369 SIGLEC5 Sialic acid-binding (g-like lectin S (CD 170) 2.45 0.0494 NM_000990 RPL27A Rbosomal protein 127a 2.32 0.0434 NM_0001019 RFS15A Rbosomal protein 127a 2.32 0.0433 NM_001019 RFS15A Rbosomal protein 1127a 2.32 0.0433 NM_001019 RFS15A Rbosomal protein 110 2.32 0.0433 NM_001019 RFS15A Rbosomal protein 110 2.30 0.0303 NM_005620 S100A11 S100 calcium-binding protein A11 (calgizzarin) 2.32 0.0441 NM_005089 NME4 Non-metastatic cells 4, protein expressed in 2.27 0.0365 NM_010108741 LOC388817 Peptidylprolyl isomerase A-like 2.23 0.0441 NM_020206	BC034271	FANCC	Eanconi anaemia, complementation group C	2.45	0.0214
NM_001006 RPS3A RP3A Rbiosomal protein S3A 2.45 0.0497 AY358369 SIGLECS Sialic acid-binding lg-like lectin 5 (CD170) 2.45 0.0449 NM_181468 ITGBH8P Integrin <i>β</i> 4-binding protein 2.40 0.0444 NM_000990 RPL27A Ribosomal protein 127a 2.32 0.0439 NM_001019 RPS15A Ribosomal protein 515a 2.32 0.0443 NM_001017 H373A H37A Ribosomal protein 515a 2.32 0.0401 NM_002107 H37A H37A H3 hintone, family 3A 2.30 0.0487 NM_005620 S100A11 S100 calcium-binding protein A11 (calgizzarin) 2.30 0.0487 NM_005089 NME4 Non-metastatic cells 4, protein expressed in 227 0.0365 0.04487 NM_0050886 ATP5E ATP synthase, H++ transporting, mitochondrial F1 complex, 4, 2.23 0.0444 NM_005089 NME4 Non-metastatic cells 4, protein expressed in 2227 0.0365 NM_0050886 ATP5E ATP synthase, H++ transporting, mitochondrial F1 complex, 223 0.04144 </td <td>NM_001997</td> <td>FAU</td> <td>Finkel–Biskis–Reilly murine sarcoma virus (FBR-MuSV)</td> <td>2.45</td> <td>0.043</td>	NM_001997	FAU	Finkel–Biskis–Reilly murine sarcoma virus (FBR-MuSV)	2.45	0.043
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	NM 001006	DDC2 V	Bibacamal protoin S2A	2.45	0.0497
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	AV250260		Sialis asid binding la like lectin 5 (CD170)	2.45	0.0497
$\begin{split} & A = A + A = A + $	NIM 101440	JIGLECJ	Static acid-binding lg-like lectin 5 (CD170)	2.40	0.0467
$\begin{tabular}{ c c c c c c c c c c c c c c c c c c c$	NM 000990		Riberand protein 1.27	2.40	0.0392
$\begin{tabular}{ c c c c c c c c c c c c c c c c c c c$	NM 002489		NADH debydrogenase (ubiquinone) L α subcomplex 4.9 kDa	2.37	0.0372
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	AK098605	EMNI2	Formin 2	2.52	0.0443
$\begin{array}{cccccccccccccccccccccccccccccccccccc$		RPS15A	Ribosomal protein SI5a	2.52	0.0401
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	NM 002107	H3F3A	H3 histone family 3A	2.32	0.0303
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	NM 005620	SIODALI	SIOO calcium-binding protein AII (calgizzarin)	2.30	0.0303
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	NM 006013	RPI IO	Bibosomal protein LIO	2.30	0.0489
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	NM 005009	NMF4	Non-metastatic cells 4 protein expressed in	2.20	0.0365
$\begin{array}{c c c c c c c c c c c c c c c c c c c $	NM 006886	ATP5E	ATP synthese H+ transporting mitochondrial EL complex	2.27	0.0171
$\begin{array}{c c c c c c c c c c c c c c c c c c c $, th SE	å subunit	2.25	0.0171
$\begin{tabular}{ c c c c c c c c c c c c c c c c c c c$	NM 001008741	100388817	Peptidylprolyl isomerase A-like	223	0 0444
$\begin{array}{c c c c c c c c c c c c c c c c c c c $	NM 032828	ZNE587	Zinc-finger protein 587	2.23	0.0487
NM_024040CUEDC2CUE domain containing 22.190.0214NM_006808SEC61BSec61 β subunit2.180.0465NM_002406MGAT1Mannosyl (α -1,3-)-glycoprotein β -1,2-N-acetylglucosaminyltransferase2.140.0415NM_002797PSMB5Proteasome (prosome, macropain) subunit, β type, 52.130.0171NM_001021RPS17Ribosomal protein S172.100.0489NM_000182HADHAHydroxyacyl-coenzyme A dehydrogenase/3-ketoacyl-coenzyme A2.070.0392thiolase/enoyl-coenzyme A hydratase (trifunctional protein), α subunit2.060.0224NM_012067AKR7A3Aldo-keto reductase family 7, member A3 (aflatoxin aldehyde reductase)2.060.043NM_005340HINT1Histidine triad nucleotide-binding protein 12.040.0444NM_145893A2BP1Ataxin 2-binding protein 1-2.440.0357NM_015503SH2B1SH2-B adaptor protein-2.080.0487NM_178033CYP4X11Cytochrome P450, family 4, subfamily X, polypeptide 1-2.040.0355CR749256XRCC2X-ray repair complementing defective repair in Chinese hamster cells 2-2.000.0357	NM 015933	HSPC016	Hypothetical protein HSPC016	2.22	0.0258
$\begin{array}{c c c c c c c c c c c c c c c c c c c $	NM 024040	CUFDC2	CUE domain containing 2	2.19	0.0214
NM_002406MGATIMannosyl (α-1,3-)-glycoprotein β-1,2-N-acetylglucosaminyltransferase2.140.0415NM_002797PSMB5Proteasome (prosome, macropain) subunit, β type, 52.130.0171NM_001021RPS17Ribosomal protein S172.100.0489NM_000182HADHAHydroxyacyl-coenzyme A dehydrogenase/3-ketoacyl-coenzyme A2.070.0392thiolase/enoyl-coenzyme A hydratase (trifunctional protein), a subunit2.060.0224NM_012067AKR7A3Aldo-keto reductase family 7, member A3 (aflatoxin aldehyde reductase)2.060.043NM_005340HINT1Histidine triad nucleotide-binding protein 12.040.0444NM_145893A2BP1Ataxin 2-binding protein 1-2.440.0357NM_015503SH2B1SH2-B adaptor protein-2.080.0487NM_178033CYP4X1Cytochrome P450, family 4, subfamily X, polypeptide 1-2.080.0435CR749256XRCC2X-ray repair complementing defective repair in Chinese hamster cells 2-2.000.0357	NM 006808	SEC61B	Sec6 β subunit	2.18	0.0465
β -1,2-N-acetylglucosaminyltransferaseNM_002797PSMB5Proteasome (prosome, macropain) subunit, β type, 52.130.0171NM_001021RPS17Ribosomal protein S172.100.0489NM_000182HADHAHydroxyacyl-coenzyme A dehydrogenase/3-ketoacyl-coenzyme A2.070.0392thiolase/enoyl-coenzyme A hydratase (trifunctional protein), α subunit α 2.060.0224NM_012067AKR7A3Aldo-keto reductase family 7, member A3 (aflatoxin aldehyde reductase)2.060.043NM_005340HINT1Histidine triad nucleotide-binding protein I 2.042.040.0444NM_145893A2BP1Ataxin 2-binding protein I 513-2.440.0357NM_015503SH2B1SH2-B adaptor protein 9450, family 4, subfamily X, polypeptide I 1-2.080.0487NM_003893LDB1LIM domain-binding I 4-ray repair complementing defective repair in Chinese hamster cells 2 -2.00-2.000.0357	NM 002406	MGATI	Mannosyl (α -1.3-)-glycoprotein	2.14	0.0415
NM_002797PSMB5Proteasome (prosome, macropain) subunit, β type, 52.130.0171NM_001021RPS17Ribosomal protein S172.100.0489NM_000182HADHAHydroxyacyl-coenzyme A dehydrogenase/3-ketoacyl-coenzyme A2.070.0392thiolase/enoyl-coenzyme A hydratase (trifunctional protein), a subunita subunit2.060.0224NM_012067AKR7A3Aldo-keto reductase family 7, member A3 (aflatoxin aldehyde reductase)2.060.0224XM_376787RPS26P10Ribosomal protein S26 pseudogene 102.060.043NM_005340HINT1Histidine triad nucleotide-binding protein 12.040.0444NM_145893A2BP1Ataxin 2-binding protein 1-2.440.0357NM_015503SH2B1SH2-B adaptor protein-2.030.0487NM_178033CYP4X11Cytochrome P450, family X, polypeptide 1-2.040.0355CR749256XRCC2X-ray repair complementing defective repair in Chinese hamster cells 2-2.000.0357	-		β -1,2-N-acetylglucosaminyltransferase		
NM_001021RPS17Ribosomal protein S172.100.0489NM_000182HADHAHydroxyacyl-coenzyme A dehydrogenase/3-ketoacyl-coenzyme A2.070.0392thiolase/enoyl-coenzyme A hydratase (trifunctional protein), \$\alpha\$ subunit2.060.0224NM_012067AKR7A3Aldo-keto reductase family 7, member A3 (aflatoxin aldehyde reductase)2.060.0224XM_376787RPS26P10Ribosomal protein S26 pseudogene 102.060.043NM_005340HINT1Histidine triad nucleotide-binding protein 12.040.0444NM_145893A2BP1Ataxin 2-binding protein 1-2.440.0357NM_015503SH2B1SH2-B adaptor protein-2.080.0487NM_015833CYP4X1Cytochrome P450, family 4, subfamily X, polypeptide 1-2.040.0355CR749256XRCC2X-ray repair complementing defective repair in Chinese hamster cells 2-2.000.0357	NM_002797	PSMB5	Proteasome (prosome, macropain) subunit, β type, 5	2.13	0.0171
NM_000182HADHAHydroxyacyl-coenzyme A dehydrogenase/3-ketoacyl-coenzyme A2.070.0392hiolase/enoyl-coenzyme A hydratase (trifunctional protein), \$\alpha\$ subunit\$\alpha\$ subunit\$\alpha\$ outputNM_012067AKR7A3Aldo-keto reductase family 7, member A3 (aflatoxin aldehyde reductase)\$\alpha\$ output\$\alpha\$ outputXM_376787RPS26P10Ribosomal protein S26 pseudogene 10\$\alpha\$ output\$\alpha\$ outputNM_005340HINT1Histidine triad nucleotide-binding protein 1\$\alpha\$ output\$\alpha\$ outputNM_145893A2BP1Ataxin 2-binding protein 1\$\alpha\$ output\$\alpha\$ outputNM_015503SH2B1SH2-B adaptor protein\$\alpha\$ output\$\alpha\$ outputNM_003893LDB1LIM domain-binding 1\$\alpha\$ output\$\alpha\$ outputCR749256XRCC2X-ray repair complementing defective repair in Chinese hamster cells 2\$\alpha\$ output	NM_001021	RPS17	Ribosomal protein SI7	2.10	0.0489
NM_012067AKR7A3Aldo-keto reductase family 7, member A3 (aflatoxin aldehyde reductase)2.060.0224XM_376787RPS26P10Ribosomal protein S26 pseudogene 102.060.043NM_005340HINT1Histidine triad nucleotide-binding protein 12.040.0444NM_145893A2BP1Ataxin 2-binding protein 1-2.440.0357NM_015503SH2B1SH2-B adaptor protein n-2.330.0487NM_178033CYP4X1Cytochrome P450, family 4, subfamily X, polypeptide 1-2.040.0355CR749256XRCC2X-ray repair complementing defective repair in Chinese hamster cells 2-2.000.0357	NM_000182	HADHA	Hydroxyacyl-coenzyme A dehydrogenase/3-ketoacyl-coenzyme A thiolase/enoyl-coenzyme A hydratase (trifunctional protein),	2.07	0.0392
XM_376787RPS26P10Ribosomal protein S26 pseudogene 102.060.043NM_005340HINT1Histidine triad nucleotide-binding protein 12.040.0444NM_145893A2BP1Ataxin 2-binding protein 1-2.440.0357NM_015503SH2B1SH2-B adaptor protein-2.330.0487NM_178033CYP4X1Cytochrome P450, family 4, subfamily X, polypeptide 1-2.080.0457NM_003893LDB1LIM domain-binding 1-2.040.0355CR749256XRCC2X-ray repair complementing defective repair in Chinese hamster cells 2-2.000.0357	NM_012067	AKR7A3	Aldo-keto reductase family 7, member A3 (affatoria aldebude reductase)	2.06	0.0224
NM_005340 HINTI Histidine triad nucleotide-binding protein I 2.06 0.0434 NM_005340 HINTI Histidine triad nucleotide-binding protein I 2.04 0.0444 NM_005340 A2BPI Ataxin 2-binding protein I -2.44 0.0357 NM_015503 SH2BI SH2-B adaptor protein -2.33 0.0487 NM_178033 CYP4X1 Cytochrome P450, family 4, subfamily X, polypeptide I -2.08 0.0487 NM_003893 LDBI LIM domain-binding I -2.04 0.0355 CR749256 XRCC2 X-ray repair complementing defective repair in Chinese hamster cells 2 -2.00 0.0357	XM 376787	RP\$26PIO	Ribosomal protein \$26 pseudogene 10	2.06	0.043
NM_145893 A2BP1 Ataxin 2-binding protein I -2.44 0.0357 NM_145893 SH2B1 SH2-B adaptor protein -2.33 0.0487 NM_178033 CYP4X1 Cytochrome P450, family 4, subfamily X, polypeptide I -2.04 0.0355 NM_003893 LDB1 LIM domain-binding I -2.04 0.0355 CR749256 XRCC2 X-ray repair complementing defective repair in Chinese hamster cells 2 -2.00 0.0357	NIM 005340		Histidine triad nucleotide binding protoin	2.00	0.043
NM_015503 SH2B1 SH2-B adaptor protein -2.33 0.0487 NM_01503 CYP4X1 Cytochrome P450, family 4, subfamily X, polypeptide I -2.08 0.0487 NM_003893 LDB1 LIM domain-binding I -2.04 0.0355 CR749256 XRCC2 X-ray repair complementing defective repair in Chinese hamster cells 2 -2.00 0.0357	NM 145893	A2RPI	Atavin 2-hinding protein 1		0.0777
NM_178033 CYP4X1 Cytochrome P450, family 4, subfamily X, polypeptide I -2.08 0.0487 NM_003893 LDBI LIM domain-binding I -2.04 0.0355 CR749256 XRCC2 X-ray repair complementing defective repair in Chinese hamster cells 2 -2.00 0.0357	NM 015503	SH2RI	SH2-B adaptor protein	-2.17	0.0337
NM_003893LDB ILIM domain-binding I-2.040.0355CR749256XRCC2X-ray repair complementing defective repair in Chinese hamster cells 2-2.000.0357	NM 178033	CYP4X1	Cytochrome P450, family 4, subfamily X, polypeptide 1	-2.05	0.0487
CR749256XRCC2X-ray repair complementing defective repair in Chinese hamster cells 2-2.010.0357	NM 003893	I DBI	LIM domain-binding 1	-2.00	0.0355
	CR749256	XRCC2	X-ray repair complementing defective repair in Chinese hamster cells 2	-2.00	0.0357

Genes differentially expressed between biopsies of good responders and poor responders. The list shows 61 annotated genes from the original list of 123 genes.

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transcription factor for osteoprotegerin (OPG), a major negative regulator of osteoclastogenesis (Khosla, 2001). In contrast, our study identified the up-regulation of ID1 in OS. As ID1 is a



Figure 3 Unsupervised clustering of 123 differentially expressed genes between good responders and poor responders. Genes were selected based on normalised data values that were greater or less in 5 good responders than those in 17 poor responders by a factor of two-fold, with statistically significant differences when grouped by 'response' according to a parametric test Welch *t*-test (*P*-value cutoff 0.05). Multiple testing correction was applied (Benjamini and Hochberg false discovery rate). Level of expression – lowest (*light green*), highest (*bright red*).

negative regulator of osteoclast differentiation (Lee et al, 2006), it is possible that the overexpression of ID1 in OS lesions may provide an explanation for OS-induced osteoclast loss. Our microarray analysis of primary OS lesions also indicates that S100A8 was profoundly reduced in OS lesions (P = 0.005; fold decrease = 100). S100A8 is highly expressed in osteoclasts in which it functions as a chemotactic-signalling molecule involved in the coupling of osteoclast and osteoblast activity (Zreigat et al, 2007). In addition, decreased expression of 15 other genes involved in osteoclast development and function (Pereira et al, 1990; Garcia et al, 1996; Lowell et al, 1996; Chertov et al, 1997; Chellaiah et al, 2000; Duong et al, 2000; Battaglino et al, 2002; Lundberg et al, 2007; Shahbazi et al, 2007; Kawano et al, 2008; Zhou et al, 2008; Soehnlein and Lindbom, 2009) was observed in the OS biopsies. Among these was CTSG, which is necessary for the recruitment of osteoclast precursors (Wilson et al. 2009b) and for the activation of MMP9, which in turn activates TGF β to enhance osteoclast activity (Wilson et al, 2009a); TYROBP/DAP12, which is essential for RANK signalling and osteoclast multinucleation and differentiation (Humphrey et al, 2004; Mocsai et al, 2004), and the NFkB subunit, RelA/p65, which promotes osteoclastogenesis by inhibiting JNK-mediated osteoclast apoptosis (Vaira et al, 2008; Soysa and Alles, 2009). We also observed decreased expression of osteoclast cellular components such as ACP5/TRAP, a classic marker of mature and active osteoclasts (Havman, 2008) and ATP6V0D1, which is found in the osteoclast membrane and critical for its resorptive activity (Xu et al, 2007). Furthermore, the decreased expression of osteoclast cellular components correlated with a decrease in the number of osteoclast cell counts in



Figure 4 Expression of highly differentially expressed genes between good and poor responders. Expression, measured as normalised spot intensity, in each of the 5 good responders and 17 poor responders of 6 highly differentially expressed genes between the two groups. *P*-value (ANOVA, Welch *T*-test, Benjamini and Hochberg false discovery rate). Bars: s.e.m.

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Figure 5 Expression of genes classically associated with chemotherapeutic response. (A) Glutathione S-transferase family, (B) DNA damage response, (C) drug transport, (D) survival, (E) apoptosis, and (F) CYP family. *P<0.05. Bars: mean + s.e.m.

immunohistochemically stained sections of OS compared with non-malignant bone. Thus, we provide evidence that OS lesions are associated with a reduction in osteoclasts.

The down-regulation of osteoclast differentiation in OS was accompanied by down-regulation of five MHC Class I and II genes belonging to the antigen-presentation pathway and of HMGB1. The correlation between MHC Class I and II deficiencies in human tumours and metastatic potential/reduced survival rates in patients is well documented (Meissner *et al*, 2005; Chamuleau *et al*, 2006; Ramnath *et al*, 2006; Seliger, 2008), whereas the interaction of HMGB1 with Toll-like receptor 4 on dendritic cells has been shown to be essential for tumour antigen processing and presentation (Apetoh *et al*, 2007). Interestingly, we observed a significant increase in the expression of five genes with overlapping functions in antigen presentation and osteoclastogenesis in the good responder group – IFI30 (P = 0.0005), TYROBP/DAP12 (P = 0.002), TMSB10 (P = 0.003), CTSB (P = 0.004), and SPP1 (P = 0.037). The IFI30 expression, in melanoma (Goldstein *et al*, 2008) and squamous cell

carcinoma (Wenzel et al, 2008), has been shown to enhance antigen presentation and to activate CTSB (Goldstein et al, 2008), which is required for osteoclast fusion during differentiation (McMichael et al, 2009). In addition, TYROBP/DAP12 is associated with active innate immune responses (Lanier, 2009) and, together with SPP1, also has a crucial function in osteoclast differentiation (Humphrey et al, 2004; Mocsai et al, 2004; Dalla-Torre et al, 2006; Inui et al, 2009). Furthermore, the expression of SPP1 in OS lesions has been correlated with improved overall survival (Dalla-Torre et al, 2006). Although there is some evidence connecting TMSB10 expression to carcinogenesis (Lee et al, 2001; Sardi et al, 2002; Alldinger et al, 2005), this is the first report linking TMSB10 expression to good chemotherapeutic response in OS. In addition, good responders had significantly higher levels of ACP5/TRAP gene expression than poor responders. Thus, these results are the first to show a possible association between antigen presentation and osteoclastogenesis in the biology of OS tumours and in their chemotherapeutic response.

This study revealed two interesting findings relating to OS chemosensitivity. First, we found that OS displayed a gene signature that was consistent with a chemoresistant phenotype. Second, genes associated with classical drug resistance were not overrepresented in chemoresistant OS lesions. These conclusions are supported by the increased expression of seven metallothionein family members in the OS biopsies. The function of metallothioneins in drug resistance has been well documented (Cherian et al, 2003; Theocharis et al, 2004; Surowiak et al, 2007), and an earlier transcriptomic study of OS reported up-regulation of MTIG and MT1L in poorly responsive tumours (Mintz et al, 2005). In this study, we found no correlation between their level of expression and response, a finding that is supported by others (Uozaki et al, 1997; Shnyder et al, 1998). Moreover, we found no correlation between good and poor responses and the expression of molecules classically associated with chemoresistance, such as GSTs and ABC transporters, or DNA damage response, apoptosis, drug metabolism, and survival genes. These results suggest that drug resistance may be a global characteristic of all osteosarcomas and that drug resistance, in chemoresistant lesions, is likely to be mediated by novel pathways.

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