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Alternative splicing of Leptin Receptor Overlapping Transcript (LEPROT) in osteosarcoma

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Keywords:	OSTEOSARCOMA, TRANSCRIPTOME, LEPROT, RNA SEQUENCING, ALTERNATIVE SPLICING, OSTEOGENIC SARCOMA
Abstract:	Alternative splicing (AS) of RNA is an essential mechanism that increases proteomic diversity in eukaryotic cells. Aberrant AS is often associated with various human diseases, including cancer. We conducted whole-transcriptome analysis of 18 osteosarcoma (OS) bone samples (paired normal - tumour biopsies). Using RNA-seq, we identified statistically significant (FDR <0.05) 26 differentially expressed transcript variants of leptin receptor overlapping transcript (LEPROT) gene. Some of the transcripts were overexpressed in normal cells, whereas others were overexpressed in tumour cells. The function of LEPROT is not completely understood. Herein, we highlight a possible association between OS and aberrant AS events and its interaction with the expression of LEPROT. We also discuss the role of LEPROT in regulating growth hormone and its receptor, and the relationship with initiation and progression of OS. This research study may help to understand the association of AS mechanism in OS and in tumorigenesis more generally. Further, LEPROT gene can also be considered as a potential biomarker of OS.

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Alternative splicing of Leptin Receptor Overlapping Transcript (LEPROT) in osteosarcoma

Short running title: **Alternative splicing of LEPROT in osteosarcoma**

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Abstract

Alternative splicing (AS) of RNA is an essential mechanism that increases proteomic diversity in eukaryotic cells. Aberrant AS is often associated with various human diseases, including cancer. We conducted whole-transcriptome analysis of 18 osteosarcoma (OS) bone samples (paired normal - tumour biopsies). Using RNA-seq, we identified statistically significant (FDR <0.05) 26 differentially expressed transcript variants of leptin receptor overlapping transcript (*LEPROT*) gene. Some of the transcripts were overexpressed in normal cells, whereas others were overexpressed in tumour cells. The function of *LEPROT* is not completely understood. Herein, we highlight a possible association between OS and aberrant AS events and its interaction with the expression of *LEPROT*. We also discuss the role of *LEPROT* in regulating growth hormone and its receptor, and the relationship with initiation and progression of OS. This research study may help to understand the association of AS mechanism in OS and in tumorigenesis more generally. Further, *LEPROT* gene can also be considered as a potential biomarker of OS.

Keywords: Osteosarcoma, transcriptome, LEPROT, RNA sequencing, alternative splicing, osteogenic sarcoma

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Impact statement

Osteosarcoma (OS, also known as osteogenic sarcoma) is the most common primary malignancy of bone in children and adolescents. The molecular mechanisms of OS are extremely complicated and its molecular mediators remain to be elucidated. We sequenced total RNA from 18 OS bone samples (paired normal - tumour biopsies). We found statistically significant (FDR <0.05) 26 differentially expressed transcript variants of *LEPROT* gene with different expressions in normal and tumour samples. These findings contribute to the understanding of molecular mechanisms of OS development and provide encouragement to pursue further research.

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Introduction

Osteosarcoma (OS) is the most common primary tumour of bone in children and adolescents, with a second peak in incidence in people over the age of 50.[1, 2] OS is characterised by the presence of malignant mesenchymal stem cells (MSCs) producing immature bone matrix or osteoid.[3] OS commonly develops in long bones such as the distal femur, proximal tibia or proximal humerus.[4] Patients usually present with pain and swelling. The diagnosis is confirmed by histology and staging studies such as MRI and systemic radionuclide scans.[5-7] Current treatment of OS, a combination of surgery and chemotherapy (doxorubicin, methotrexate, and cisplatin), has improved outcomes significantly, although this regime is still only successful in 90% of patients.[8] The precise underlying mechanism of OS remains obscure in most individuals, however, the risk of developing OS is influenced by bone turnover, age, environment (drug or radiation therapy), other bone diseases (such as Paget's disease), genetic alterations and hormonal regulation of puberty (such as growth hormone).[9-13]

RNA sequencing (RNA-Seq) technology is a powerful tool to analyse the transcriptome of a cell.[14] Gene expression studies have been traditionally carried out by northern blot and quantitative polymerase chain reaction (qPCR), which are limited to single transcript expressions.[15, 16] The RNA-seq technique provides higher resolution of the dynamic nature of the transcriptome.[15, 17] Beyond measuring gene expression, this technique facilitates the discovery of novel transcripts, identification of alternative (differential) splicing (AS) events in genes and detection of allele-specific expression on a genome-wide scale.[15-18] Consequently, RNA-seq is a useful method for interpreting the functional elements of the genome and also understanding the underlying mechanisms of complex diseases in a species.[19, 20] AS is a crucial mechanism that generates proteomic diversity in eukaryotes.[19] It is mainly regulated by cis-acting elements and

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3 trans-acting factors.[21] Growing evidence suggests that more than 90% of multi-exon genes
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5 undergo AS in humans.[22, 23] Deregulation of AS programming can produce a variety of transcript
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8 isoforms with unique protein-coding and possibly different or antagonistic biological functions
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10 from the same gene.[24, 25] Predictably, abnormal splicing can disturb normal cellular physiology
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13 and eventually lead to diseases, including cancer.[23-26] The involvement of AS in each of the
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15 widely recognised and accepted hallmarks of cancer has been investigated.[27] In particular,
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17 apoptosis and metastasis are directly affected by AS.[28] Moreover, it can also play an essential
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20 role in invasiveness, angiogenesis and chemo/radio-resistance to therapy.[28, 29]
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22 Little is known about the underlying molecular mechanism of AS in various cancer types,
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25 especially in OS. To improve our understanding of the genetic mechanisms involved in
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27 developing OS, we performed RNA-Seq transcriptome analysis from 18 normal - tumour pairs of
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30 bone biopsies by computational bioinformatics. Differential exon usage was studied to highlight
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32 AS and transcription events present between normal and tumour samples using the DEXSeq
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35 package. Another aim of this study was to identify potential molecular biomarkers and novel
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37 therapeutic target candidates for the early detection and treatment of OS.
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39 **Materials and Methods**
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42 Samples collection
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45 The study was investigated and approved by the Ethics Review Committee on Biomedical
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47 Research of Hue University of Medicine and Pharmacy. The participants and patient
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50 representatives signed and dated the informed consent forms before surgery.
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52 Normal and cancerous bone samples were collected directly after surgery (limb sparing or
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55 amputation) from eighteen Vietnamese patients who, previously, had histological confirmation
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of OS. The collected biopsies were replaced on dry ice and stored at -80°C until processed for RNA extraction.

Total RNA isolation from bone biopsies

Approximately fifty milligrams of bone sample was ground with liquid nitrogen in a pestle and mortar and pre-TRIzol treatment was performed (Invitrogen Corp., Carlsbad, CA, USA). RNeasy Fibrous Tissue Mini Kit (Qiagen Inc., Valencia CA, USA) was used to isolate total RNA from bone tissue following the manufacturer's protocol. Isolated RNA was completely dissolved in RNase-free water and stored at -80°C. The quality of total RNA was determined by Agilent 2100 Bioanalyzer system and the RNA 6000 Nano Kit (Agilent Technologies Inc., CA, USA).

Fifty nanograms of total RNA was amplified using Ovation RNA-Seq System V2 (NuGen, Emeryville, CA, USA) and the resulting cDNAs were pooled in equal amounts. The pooled cDNA was used to prepare the DNA fragment library using SOLiD System chemistry (Life Technologies Corp, Carlsbad, CA, USA). Sequencing was done by SOLiD 5500W platform and DNA sequencing chemistry (Life Technologies Corp., Carlsbad, CA, USA). Raw data (75 bp) were colour-space mapped to the human genome hg38 (GRCH38) reference through the mapping algorithm implemented in the Lifescope™ Genomic Analysis software (Life Technologies Corp.). The mapping confidence had been noted as higher than 90 due to the quality threshold was set to 10. The reads scores below than 10 were filtered out. Mapping quality was at average of 30. Annotation of the reads based on the gencode v22 files.[30]

Statistical data analyses were performed through the DEXseq package for R.[31] DEXseq is a Bioconductor package specifically designed to find differential exon usage (DEU) based on

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RNA-seq data. DEU describes the relative usage of exons induced by the experimental conditions.[32]

More details on data pre-processing can be found here (https://bioconductor.org/packages/release/bioc/vignettes/DEXSeq/inst/doc/DEXSeq.html). The DEXseq package also performs sample comparison and adjusts the P-value to overcome multiple testing problems.[33-35] Through DEXseq package, the Benjamini-Hochberg procedure used to control the false discovery rate (FDR).[36]

Results

Patient characteristics

Paired samples (normal - tumour) of surgical biopsies of bone were collected from 18 Vietnamese OS patients. Diagnosis was confirmed by a pathologist by examination of the biopsies. Tumour sites were located in the femur (55.56%), tibia (33.33%) and humerus (11.11%). Patient age ranged from 7 to 52 years (mean=18 years) and there were 7 females and 11 males. Patients' demographic and clinical characteristics are listed in **Table 1**.

Validation of differential exon usage of LEPROT in patient samples

A Mean-Average (MA)-plot of the log2 fold change of all transcripts (over 4075 transcripts) expressed in normal and tumour samples is presented in **Figure 1**. Red points indicate the log fold change versus average normalised count per exon (FDR < 0.05), whereas red triangles represent exons expressed with log2-fold change greater than 2 or less than −2.

The purpose of the study was to investigate AS events between 18 paired normal-tumour OS samples by RNA-seq. As shown in **Figure 2A**, statistically significant (FDR < 0.05) changes in

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expression levels of Leptin Receptor Overlapping Transcript (*LEPROT*) exons were observed between normal and tumour samples. Most of the exons are expressed in normal tissue, while expression in the tumour sample remained low. However, DEXseq removes the exon level changes in expression to highlight differential exon usage of the gene. The exons shown in purple presented with significant differential exon usage (bottom panel, bin E014, E040 and E042), suggesting potential AS events (**Figure 2B**). The comparisons of each exon expression between tumour and normal samples were performed by normalisation counts, shown in **Figure 2C**.

We identified 27 novel transcript variants of the *LEPROT* gene (**Table 2**), 26 of those transcripts have statistically significant ($FDR < 0.05$) different exon usage. This suggests that different isoforms use the exons differently. One transcripts (E037) identified as having different exon usage was subsequently shown to be non-significant ($FDR > 0.05$, highlighted in red in **Table 2**).

As visualised in **Figure 2A** and listed in **Table 2**, 5 transcripts of *LEPROT* demonstrated overexpression in the tumour samples, while 22 transcripts (21 significant / 1 non-significant) presented with overexpression in the normal samples. Decrease expression of *LEPROT* transcripts in tumour samples eventually downregulates general LEPROT expression in tumour samples.

Discussion

The limiting factor in improving treatment for OS is a lack of understanding of the molecular mechanism of the disease, which leads to an inability to diagnose and treat OS at an earlier stage, or to develop more effective therapies. RNA-seq can be a potentially powerful method to identify differential exon usage in OS and allow identification of potential biomarkers of the

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disease.[27] Differential usage of exons produces different transcripts from the same gene.[18] Cancerous cells can have alternate patterns of exon usage within individual genes compared to normal cells, suggesting that AS may play a key role in shaping the phenotype of the tumour.[23]

In this study, we have investigated AS events in 18 paired normal -tumour OS samples. Interestingly, our results showed that the *LEPROT* gene underwent AS and identified 26 statistically significant (FDR < 0.05) novel transcripts. These transcripts showed different expression levels between tumour and normal tissues, suggesting there is a potential functional association between AS of *LEPROT* and cancer progression.

A genome-wide expression analysis has suggested an association between *LEPROT* regulation and apoptosis pathway.[37] Unfortunately, the study did not explain the association further. However, the manifestations of *LEPROT* gene mutations have not been extensively studied, resulting in an incomplete understanding of the function or dysfunction of this gene in cancer metabolism.[38]

LEPROT is expressed widely in many human tissues, but does not appear to be expressed in tumours.[39] Therefore, loss or frequent downregulation of *LEPROT* expression could be associated with tumour formation. The cause of downregulation of the *LEPROT* gene in cancer cells remains unknown. One study has suggested that AS can downregulate overall gene expression by tagging specific transcripts for degradation through the nonsense-mediated mRNA decay (NMD) surveillance pathway.[40] NMD frequently controls the translation stage and degrades the transcripts if they contain a premature termination codon (PTC), which can arise from genetic mutations or AS.[41, 42] Truncated proteins cannot function properly or could even be toxic to the body, therefore, the synthesis of truncated proteins can contribute to various cancer types.[43] We therefore encourage further investigation of the regulation of *LEPROT* gene

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expression in normal and tumour cells. Our analysis highlighted *LEPROT* transcripts as being significantly under-expressed in 21 out of 26 tumour samples. Decrease expression of *LEPROT* transcripts in tumour samples, eventually can lead to reduction of total *LEPROT* expression level. The 5 overexpressed transcripts (absolute value of $\log_2$ fold-change > 1) can be considered as the noise from overall downregulation of *LEPROT* gene in OS. Therefore, it can be argued that they are not specific to OS initiation and progression. LEPROT is encoded by LEP receptor (*LEPR*) gene and they share the first two 5'-UTR exons.[44] Unsurprisingly, we previously identified significant downregulation of *LEPR* in OS.[30]

Several studies have discussed the association between *LEPROT* expression level and growth hormone (GH) activity.[44] GH stimulates the development of bone and cartilage in children and adolescents.[45] It also has been associated with induction of Insulin-like growth factor 1 (*IGF-1*) and IGF-1 receptor (*IGF-1R*) gene expression, alterations in glucose metabolism and modulation of cell proliferation genes.[46-48] *IGF-1* is produced in the liver, stimulated by GH, and plays a main role in tissue growth and development.[49-52] Higher circulating levels of *IGF-1* have a profound impact on cell proliferation, differentiation, promotion of cellular longevity and inhibition of apoptosis.[53, 54] Consequently, overexpression of *IGF-1/IGF-1R* have been implicated in tumour formation, angiogenesis and metastasis in various human cancer types, including OS.[46-48, 53] According to one study, *LEPROT* is directly involved in a receptor-mediated cell signalling pathway by regulating cell surface expression of growth hormone receptor (GHR) at the molecular level. The same study also suggested that silencing of *LEPROT* increases cell-surface expression of GHR in a mouse model.[55] Thus, increasing GHR expression level will lead to higher circulating levels of *IGF-1* and may eventually result in tumorigenesis.[47-49, 53, 55]

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Dysregulation of *LEPROT* is associated with various bone inflammation diseases in humans through the key inflammatory cytokines such as tumour necrosis factor alpha (*TNF α*) and interleukin 6 (*IL-6*). Several studies have highlighted that bone inflammation is a frequent precursor to initiation and progression of OS.[56] Further, OS cells promote local inflammation and this leads to activation of local immune responses.[57] Not surprisingly, upregulation of *IL-6* and *TNF-α* have been observed in human OS cells.[58, 59] In addition, *IL-6* mediates tumour-host interactions that facilitate lung colonisation of metastasis-initiating by OS cells.[60, 61] Consequently, *LEPROT* may be implicated in OS initiation and metastasis through upregulating expression of *IL-6* and *TNF-α*.

In conclusion, our data provide strong evidence that AS events of the *LEPROT* gene may be a risk factor for OS, and downregulation of *LEPROT* can be associated with tumorigenesis in bone. These results may facilitate a better understanding of the underlying molecular mechanism related to the initiation and progression of OS, thereby allowing identification of effective novel molecular target candidates for the treatment of the disease.

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Authors' Contributions

ER wrote the manuscript. XDH contributed in samples collection and laboratory works. JX provided valuable opinions related to the study. AM and KM participated in design of the experiments. DW and SK supervised the experiments. All authors revised the manuscript.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical Approval

The Ethics Review Committee on Biomedical Research of Hue University of Medicine and Pharmacy approved the protocols and informed consent form elaborated in the study. The informed consent forms signed and dated by the participants and representatives of patients.

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Figure Legends

Figure 1. Mean-Average (MA)-plot of normalised mean expression versus log2-fold change. The red marks represent the log of fold change versus average normalised count per exon that are significant (FDR <0.05). The red triangles represent exons with log2-fold change greater than 2 or less than −2. The grey marks and triangles represent non-significant results.

Figure 2. DEXseq analysis on RNA-Seq data. The x -axis shows individual exons within *LEPROT* gene, whereas the y-axis represents exon expression (A), exon usage (B) and normalisation counts (C). The bars below the x -axis represent exons. The lines between the exons are introns. The numbers at the bottom are genomic locations of *LEPROT*. A highlights the fitted expression values of each of the exons of *LEPROT* gene for normal (red) and tumour (blue) samples. B visualises exon usage in the gene. The exon in purple (E014, E040 and E042) and 26 purple lines between the bars and exon names indicate a significant differential exon usage (FDR<0.05), whereas E037 revealed insignificant splicing (FDR>0.05). C shows the comparison of exon expression between the samples.

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Table 1. Characteristics of osteosarcoma patients in the present study (n= 18).

Patient Code	Gender	Age	Site of tumour	Stage	Metastasis	Chemotherapy
OSVN001	Female	16	Femur	I	No	Yes
OSDN001	Male	23	Tibia	III	Yes	Yes
OSVN003	Male	13	Femur	I	No	Yes
OSVN004	Female	16	Femur	I	No	Yes
OSVN005	Male	18	Femur	I	No	Yes
OSVN006	Male	18	Femur	I	No	Yes
OSVN008	Male	52	Femur	I	Yes	No
OSHN008	Female	24	Tibia	I	No	Yes
OSHN009	Male	16	Femur	I	No	Yes
OSHN010	Female	20	Femur	I	No	Yes
OSHN011	Male	7	Tibia	I	No	No
OSHN012	Male	11	Humerus	I	No	No
OSHN013	Male	17	Femur	I	No	Yes
OSHN014	Female	16	Tibia	III	No	Yes
OSVN015	Male	15	Tibia	I	No	Yes
OSHN015	Female	8	Tibia	I	No	Yes
OSHN016	Male	20	Femur	I	No	Yes
OSHN017	Female	23	Humerus	I	No	Yes

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Table 2. Differently spliced transcript variants of *LEPROT*.

Group ID: Exon ID	Normal *	Tumour *	log2(fold-change) Normal/Tumour	p value	padj	Genomic Data Start	Genomic Data End	Genomic Data Width
ENSG00000213625.7+ENSG00000116678.17: E014	71.55	214.78	-1.59	3.03E-13	4.07E-08	65431803	65431920	118
ENSG00000213625.7+ENSG00000116678.17: E015	88.50	242.77	-1.49	2.74E-10	9.22E-06	65431921	65432064	144
ENSG00000213625.7+ENSG00000116678.17: E027	45.21	28.69	0.66	3.32E-09	4.25E-05	65608753	65608901	149
ENSG00000213625.7+ENSG00000116678.17: E032	46.70	27.21	0.78	4.39E-09	4.73E-05	65617964	65618146	183
ENSG00000213625.7+ENSG00000116678.17: E016	498.02	1229.84	-1.30	1.01E-08	7.57E-05	65432065	65436007	3943
ENSG00000213625.7+ENSG00000116678.17: E022	21.01	13.80	0.61	8.97E-09	7.57E-05	65596448	65596593	146
ENSG00000213625.7+ENSG00000116678.17: E036	17.21	10.89	0.66	1.10E-08	7.57E-05	65622906	65622981	76
ENSG00000213625.7+ENSG00000116678.17: E021	21.80	12.71	0.78	9.81E-09	7.57E-05	65592657	65592865	209
ENSG00000213625.7+ENSG00000116678.17: E019	33.89	20.33	0.74	1.29E-08	8.44E-05	65570473	65570802	330
ENSG00000213625.7+ENSG00000116678.17: E040	586.05	394.12	0.57	1.75E-08	0.00010921	65633152	65635409	2258
ENSG00000213625.7+ENSG00000116678.17: E009	6.97	19.68	-1.50	2.13E-08	0.00011916	65425303	65425378	76
ENSG00000213625.7+ENSG00000116678.17: E028	51.71	31.11	0.73	3.28E-08	0.00013701	65609947	65610106	160
ENSG00000213625.7+ENSG00000116678.17: E023	27.34	17.71	0.63	1.37E-07	0.00037999	65598660	65598804	145
ENSG00000213625.7+ENSG00000116678.17: E024	78.79	53.17	0.57	3.83E-07	0.00072278	65601392	65601682	291
ENSG00000213625.7+ENSG00000116678.17: E031	53.74	33.47	0.68	3.83E-07	0.00072278	65616008	65616224	217
ENSG00000213625.7+ENSG00000116678.17: E012	45.95	125.95	-1.45	4.18E-07	0.000759	65429931	65430048	118
ENSG00000213625.7+ENSG00000116678.17: E034	31.50	20.95	0.59	3.14E-06	0.00236337	65619940	65620023	84
ENSG00000213625.7+ENSG00000116678.17: E035	30.34	19.92	0.61	3.24E-06	0.00240758	65621353	65621458	106
ENSG00000213625.7+ENSG00000116678.17: E042	17.90	10.30	0.80	5.92E-06	0.00325498	65636191	65637102	912
ENSG00000213625.7+ENSG00000116678.17: E029	27.13	19.09	0.51	6.46E-06	0.00347045	65610214	65610268	55
ENSG00000213625.7+ENSG00000116678.17: E033	14.09	9.19	0.62	6.61E-06	0.00352845	65619928	65619939	12
ENSG00000213625.7+ENSG00000116678.17: E020	15.80	10.90	0.54	9.26E-06	0.00429905	65572326	65572449	124
ENSG00000213625.7+ENSG00000116678.17: E025	26.93	15.93	0.76	1.14E-05	0.00479061	65601843	65601960	118
ENSG00000213625.7+ENSG00000116678.17: E026	47.90	32.81	0.55	1.19E-05	0.00490364	65605038	65605237	200
ENSG00000213625.7+ENSG00000116678.17: E018	8.83	4.67	0.92	1.83E-05	0.00607585	65565546	65565605	60
ENSG00000213625.7+ENSG00000116678.17: E030	21.85	15.65	0.48	4.28E-05	0.00984627	65610269	65610296	28
ENSG00000213625.7+ENSG00000116678.17: E037	11.05	5.21	1.08	0.000784	0.05035195	65622982	65623384	403

***Normalised counts**

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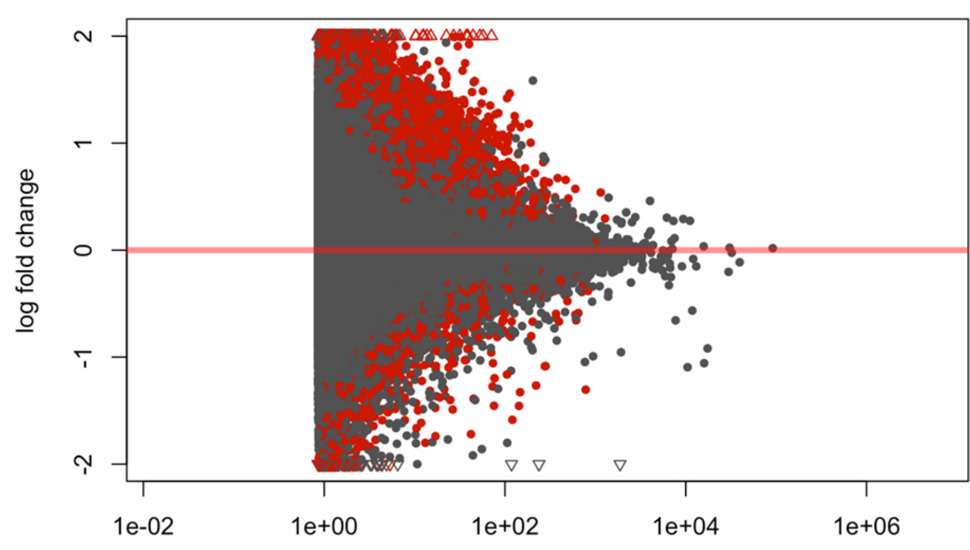
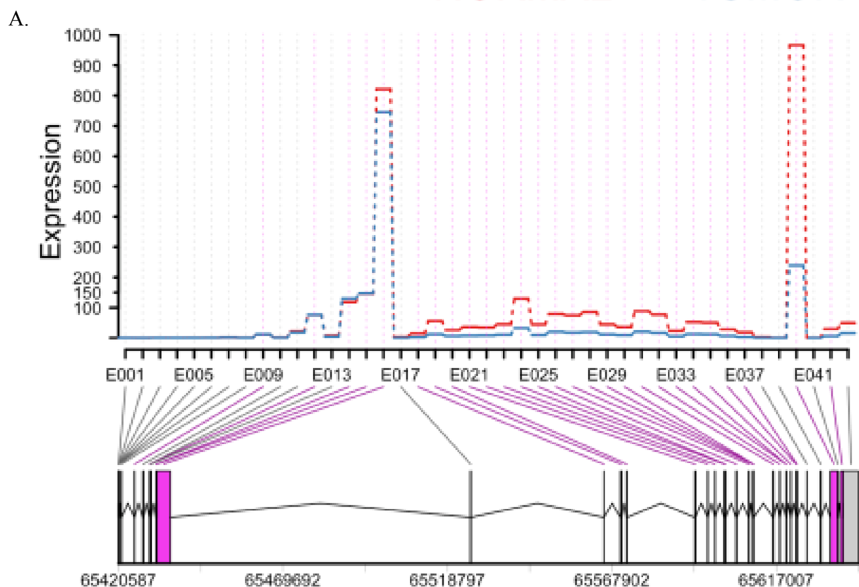


Figure 1. Mean-Average (MA)-plot of normalised mean expression versus log2-fold change.

250x150mm (96 x 96 DPI)

NORMAL TUMOR

A.



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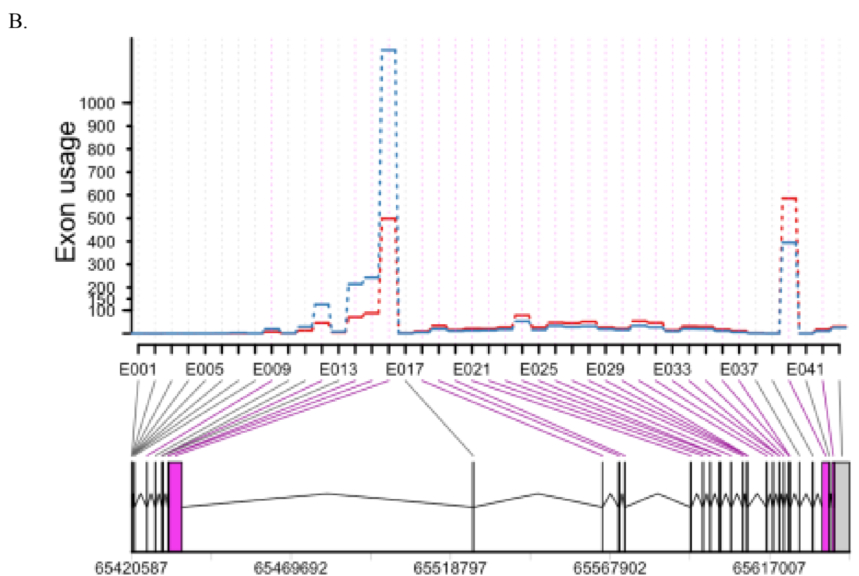

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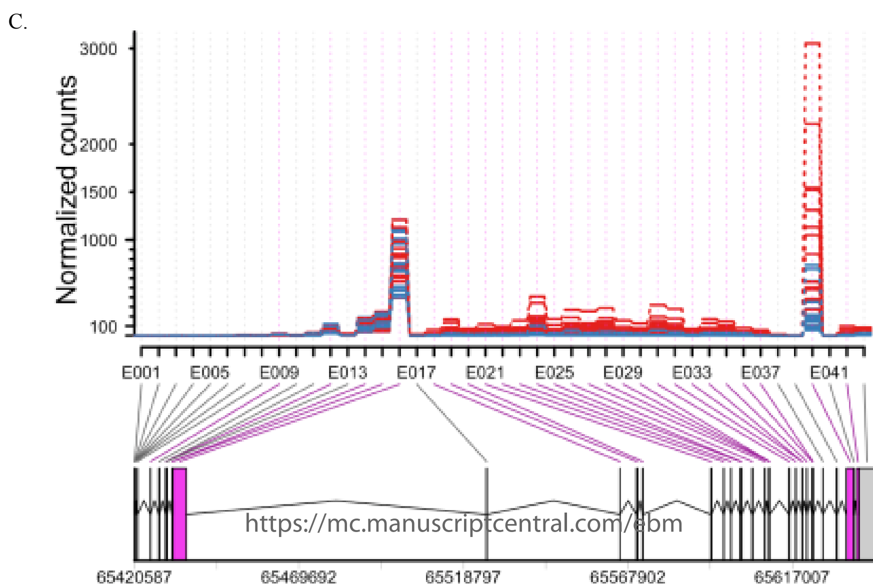

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