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Analysis of transcripts from 17p13.3 in medulloblastoma suggests *ROX/MNT* as a potential tumour suppressor gene

Ales Cvekl Jr. [a], Jiri Zavadil [b,1], Barbara K. Birshtein [c], Michael A. Grotzer [d], Ales Cvekl [a,e,*]

[a] *Department of Ophthalmology and Visual Sciences, Albert Einstein College of Medicine, Bronx, NY 10461, USA*
[b] *Department of Medicine, Albert Einstein College of Medicine, Bronx, NY 10461, USA*
[c] *Department of Cell Biology, Albert Einstein College of Medicine, Bronx, NY 10461, USA*
[d] *Division of Oncology, University Children's Hospital, Zurich, Switzerland*
[e] *Department of Molecular Genetics, Albert Einstein College of Medicine, 909 Ullmann, 1300 Morris Park Avenue, Bronx, NY 10461, USA*

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Abstract

Haploinsufficiency of the human 17p13.3 region is associated with 35% to 50% of medulloblastomas, indicating the presence of one or more tumour suppressor genes which have not yet been identified. Of 119 genes residing in this region, seven genes- *14-3-3ε* (*YWHAE*), *HIC-1*, *ROX/MNT* (a helix–loop–helix transcription factor and member of the MYC/MAX superfamily), *KIAA0399*, *UBE2G1* (ubiquitin ligase), *ALOX15*, and *MINK* – encode proteins with potential links to cancer. We investigated these genes and found significant levels of expression of ROX/MNT in adult human cerebellum, and in embryonic and postnatal mouse cerebellum. Six of 14 medulloblastomas showed a reduction of ROX/MNT expression, accompanied by a reduction of both UBE2G1 and 14-3-3ε in three tumours and a reduction of UBE2G1 in one tumour. Moreover, the relative expression of MYC to ROX/MNT was increased in 4 of the 14 medulloblastomas. Collectively, these data suggest that *ROX/MNT* should be considered a potential tumour suppressor gene in medulloblastoma.

Keywords: Medulloblastoma; *ROX/MNT*; *c-MYC*; Quantitative RT-PCR

1. Introduction

Medulloblastomas (MBs) constitute more than 20% of all paediatric brain tumours and are the most common malignant brain tumours in children [1]. Medulloblastomas are also known as primitive neuroectodermal tumours (PNET) of the cerebellum, and are clinically characterised as aggressive tumours with high risk of metastasis and resistance to current non-specific therapeutic approaches. Better understanding of the aetiology of this cancer is essential to advance treatment.

Recent molecular studies of medulloblastomas have focused on four major areas, including the cellular origin of malignant cells, identification of genetic changes associated with medulloblastomas, identification of genes deregulated in medulloblastomas, and the potential to promote apoptosis of tumour cells as a treatment for this malignancy. The primary cells forming MB have been recently identified as cerebellar granule cell precursors (GCPs) [2]. Granule cells compose most of the

Abbreviations: Medulloblastoma, MB; Primitive neuroectodermal tumour, PNET; Tumour suppressor gene, TSG; Quantitative (real time) PCR, qPCR.

* Corresponding author. Tel.: +1 718 430 3217; fax: +1 718 430 8778.
E-mail address: cvekl@aecom.yu.edu (A. Cvekl).
[1] Present address: Department of Pathology, NYU School of Medicine, New York, NY 10016, USA.

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cerebellum and the brain, originating from neural crest cells, from which the differentiation of most neural cells commences (for a Review, see [3]). Early specification of neural crest cells leads to neural precursor cells. During late embryogenesis, neural precursor cells exit the rhombic lip, a dorsal hindbrain structure, where they are initially manufactured, and migrate to the site of early cerebellar development where they form the external germinal layer. GCPs are reminiscent in morphology to MB cells. MB forms in the ectoderm of the cerebellum before GCPs commence final differentiation into granule cells during subsequent involution into the cerebellum's endoderm [3]. The expression of markers of the terminally differentiated state, such as neurotrophil alkaline phosphatase (β-NAP), basic helix–loop–helix transcription factor NSCL1, and neurotrophin-3 tyrosine kinase receptor TrkC, are associated with a favourable clinical prognosis of MB [2].

Haploinsufficiency, i.e. the loss of one allele of a gene, of 17p13.3 genes is found in 35–50% of medulloblastomas [4]. Deletion of this region is also found in brain, breast, lung, and ovarian cancers, supporting the idea that the 17p13.3 region contains one or more tumour suppressor genes (TSG). Haploinsufficient TSGs are often coupled with a mutation in the remaining allele, thereby further reducing adequate gene expression. Identification of candidate tumour suppressor genes (TSG) residing in 17p13.3 is critical for the development of mouse models for medulloblastomas and for identifying pathways affected by the haploinsufficiency that may trigger cancer formation. If such a gene were involved in either cellular differentiation and/or apoptosis, focus on the respective pathway could provide an opportunity for therapeutic intervention [5–7].

Tumour formation is characterised by genomic instability resulting in the deregulation of numerous genes. Deregulation of genes encoding transcription factors, including BMI1, MATH1, PAX5, and components of signal transduction pathways, including PCT2 and SFRP1, which are involved in cerebellar development, appears to be central to MB tumorigenesis [8–10]. The present study supports the idea that at least one TSG candidate resides within 17p13.3 as 6 of 14 tumours analyzed here exhibited reduced transcript levels of ROX/MNT, a member of the MYC/MAX family of regulators of essential cellular and developmental processes.

2. Patients and methods

2.1. Primary tumour samples

Frozen tumour tissue that was adequate (>90% tumour tissue) to perform reverse transcriptase-polymerase chain reaction (RT-PCR) was available from 14 MB patients (seven male and seven female) treated at the University Children's Hospital of Vienna, Austria ($n = 13$), and the University Children's Hospital of Zurich, Switzerland ($n = 1$). Isolation of total RNA and preparation of cDNA was performed as described earlier [6]. All diagnoses were confirmed by a histological assessment of the tumour specimens obtained at surgery by experienced neuropathologists. The median age at diagnosis for these MB patients was 9.1 years (range 1.1–32.3 years).

2.2. Analysis of gene expression profiles of 119 genes residing within 17p13.3

A total number of 119 genes in 17p13.3 are displayed by LOCUSLINK (http://www.ncbi.nih.gov/LocusLink/list.cgi?*Q*=17p13.3&ORG=&*V*=0), a site used by the Human Genome Project. Online Mendelian of Man (OMIM, www.ncbi.nlm.gov/OMIM) was used to search all presently known and candidate TSGs [11]. Basic local alignment search tool (BLAST) nucleotide searches were done to retrieve any 17p13.3 genes whose nucleotide sequence was similar to any TSG nucleotide sequence. Three servers were employed – NCBI (www.ncbi.nih.gov), SWISSPROT (www.expasy.ca/swissprot), and Bioinbgu (www.cs.bgu.ac.il/~bioinbgu/form.html). 17p13.3 genes whose protein sequences were 40% or more identical to known TSGs and/or 50% similar to known TSGs were considered to have an increased chance of having a function similar to that of a TSG. A Unigene database (www.ncbi.nlm.nih.gov/unigene) was utilised to examine the expression profiles of each gene within 17p13.3. Genes found in certain tissues and stages of development (i.e., embryonic and neonatal brain, cerebellum, primitive neuroectoderm, and cell lines derived from brain tumours) qualified as potential TSGs associated with medulloblastomas.

2.3. Human cerebellum and mouse brain cDNAs

Total RNA from one human cerebellum was obtained from Ambion (Woodlands, TX) and converted into cDNA using oligo dT primer and SuperScript II reverse transcriptase (Invitrogen, Gaithersburg, MD), according to the manufacturer's instructions. Mouse cDNA from embryonic (E14.5) hindbrain/rhombencephalon, postnatal day 7 (D7) cerebellum, and adult week 5 (D35) cerebellum were obtained from a mouse brain expression profiling kit (Origene Technologies, Rockville, MD). All samples were stored at −20 °C.

2.4. Quantitative (real-time) PCR

Real-time quantitative PCR (qPCR) using SYBR® Green Master Mix (Applied Biosystems, Foster City, CA) normalised to β2 microglobulin (B2M) and β-actin (ACTB) was used to investigate whether mRNA from any of the genes was expressed at reduced amounts in

Table 1
Primer sets of each gene in human and mouse used quantitative polymerase chain reaction (qPCR)

	Forward	Reverse	Product size
Human			
ALOX15	GCCAGCATGAGGAGGAGTAT	CAATTTCCTTATCCAGGGCA	97
HIC1	GACTTTTCCTGAAGCGGACA	CAGCAGCTGCCTGGAGTG	100
KIAA0399	CTTCTTCGTCACCGAGAACC	GGCCTTGTTTGGGTAGTTGA	82
MINK	AGTTCCTGTGTGAGCGGAAT	CCAAGGCCACTACATTGAGG	51
ROX MNT	GACGAGGATATGGAGGAGGA	GACGATGGCTCAGCTTAGGT	58
UBE2G1	TGGCAGACCCTAATGGAGAC	GGTGGGTAGAGTGCAGGAAA	93
14-3-3ε	GGCGAGTCCAAGGTTTTCTA	CGTTTCCTGTGGCAAATTCT	76
MYC	CCCTCAACGTTAGCTTCACC	CACCGAGTCGTAGTCGAGGT	53
B2M	TGCTGTCTCCATGTTTGATGTATCT	TCTCTGCTCCCCACCTCTAAGT	54
ACTB	CTGGAACGGTGAAGGTGACA	AAGGGACTTCCTGTAACAATGCA	50
Mouse			
ALOX15	CTGAGATTGGGTTGCAAGGT	TCAGGGTTTGGGAAGTGTTC	77
HIC1	AGACGATGCTGGACACGAT	AGCAGTAGCTGCCTCGAATG	52
KIAA0399	TACCTGCTGGCCTTAACCAC	GTTGATGGCCAGGCTGATAG	90
MINK	CTCAAGTTCCTGTGTGAGCG	TAAACTTGGCTGCTTCCTCC	74
Rox/Mnt	TGGCTTACGTTCTCTGCCTT	ATAGCAGCAACAGCACAGGA	50
UBE2G1	AAGCCAAGAAACTGCTTTTGAG	TGCAGGAAAAACAGTGCCAT	100
14-3-3ε	ATAACCTGACGCTGTGGACC	CCTGCAGCGCTTCTTTATTC	69
B2M	TGCAGAGTTAAGCATGCCAG	CAAATGAATCTTCAGAGCATCA	50
HPRT1	CTGGTGAAAAGGACCTCTCG	CAAGGGCATATCCAACAACA	53

the tumour masses compared with the normal cerebellum [12]. Nineteen pairs of primers were designed by Primer-3 Old Version on-line software (www.basic.nwu.edu/biotools/Primer3.html) (see Table 1). The PCR mix consisted of 2 μl of primers (1.8 μM each), 2 μl of cDNA (approximately 2 ng), and 4 μl of 2×SYBR® green mix. Amplification conditions comprised of a 15 s denaturation step at 95 °C followed by a 1 min annealing/elongation step at 60 °C for 40 cycles using ABI model 7900HT Sequence Detection System (Foster City, CA). Relative quantities were measured by determining the fluorescence emitted by the cDNA [13]. Titrations of diluted cDNA (1:10 and 1:100) were performed a priori to ensure linearity of the amplification. Dissociation curves were collected to control the quality of amplification. All experiments were performed twice in triplicate. The median value for each threshold cycle (C_T) was used for the calculations. The median of the hexaplet PCR measurements was used to express the relative abundance of each mRNA in a single and robust representative value, and standard deviations were calculated across replicate amplifications to indicate pipetting error, ranging from 5% to 18% on a linear scale. On the log scale, these standard deviations were omitted as they were incompatible with the logarithmic conversion.

3. Results

3.1. Database searches to generate a list of potential TSGs residing in a region of 17p13.3

The human genome project predicted the location of 119 genes within 17p13.3. Many of these genes encode olfactory receptors and, as such, were dismissed as potential TSGs. Evaluation of the structural and functional relationships of the remaining group of 103 genes to known TSG and cancer-related pathways, and their expression pattern in the cerebellum and/or brain led us to identify a group of seven potential TSGs, 14-3-3ε, *HIC*−1, *ROX*/*MNT*, *KIAA*0399, *UBE*2*G*1, *ALOX*15,

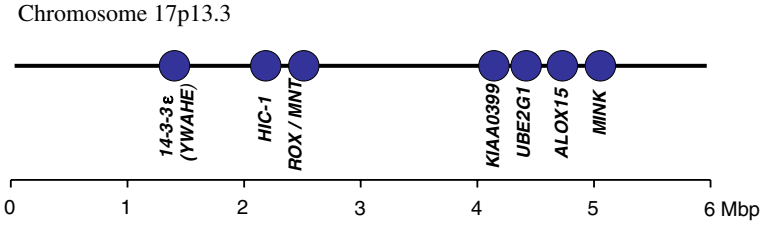

Fig. 1. Schematic representation of human chromosome 17p13.3 and localisation of seven genes including *14*−*3*−*3ε*, *HIC*−1, *ROX*/*MNT*, *KIAA*0399, *UBE*2*G*1, *ALOX*15 and *MINK*, analysed here.

Table 2
Relative expression levels of seven genes from 17p13.3 compared with β-actin (*ACTB*) in adult human cerebellum

Gene	Relative expression
ALOX15	0.005
HIC-1	0.002
KIAA0399	0.013
MINK	0.002
ROX/MNT	0.095
UBE2G1	5.565
14-3-3ε	17.121

and *MINK* (see Fig. 1), which are located across the entire region of 17p13.3.

3.2. Expression of seven genes from 17p13.3 in adult human cerebellum and in microdissected mouse brain

Expression of these seven potential TSGs in 17p13.3 was assessed in adult human cerebellum and in microdissected mouse brain. Transcripts encoding γ-actin (*ACTB*) were used for normalisation of the human data samples. The results, shown in Table 2, revealed that significant expression was observed only for 14-3-3ε, ROX/MNT and UBE2G1. Although ROX/MNT expression was approximately 180-weaker than 14-3-3ε, and 58-times weaker than UBE2G1, ROX/MNT expression was 48-times higher compared with HIC-1 and MINK, 19-times higher than ALOX15 and 7-times higher than that of KIAA0399.

Next, we compared the relative levels of expression of ROX/MNT and 14-3-3ε to hypoxanthine guanine phosphoriboysyl transferase 1 (HPRT1) in the mouse at embryonic day 14.5 (E14.5) hindbrain (an embryonic part of the central nervous system from which the cerebellum develops), day 7 (D7) and day 35 (D35) cerebellum. The frontal and posterior cortex were examined for comparison. The results (see Fig. 2) demonstrate that ROX/MNT expression is maintained from D7 to D35 cerebellum. In contrast, in both subregions of the cortex, the ROX transcript level dropped significantly (Fig. 2). There was a similar gradual decrease in 14-3-3ε transcripts from embryonic stage E14.5 to adult stages in all three regions examined. These data showed that ROX/MNT is transcribed throughout cerebellar development. Maturation of human and mouse cerebellum has been shown to be very similar [3]. Hence, human cerebellum development is likely to require a similar pattern of ROX expression.

3.3. Expression analysis in medulloblastomas

The expression of the candidate TSGs in 17p13.3 was measured in 14 samples of medulloblastomas, compared with control genes *B2M* and *ACTB*, and normalised against medulloblastoma sample MB1. The results, based on six experimental points, are shown in Fig. 3. Inspection of the profiles revealed that ROX/MNT expression in 6 samples (MB3, 5, 7, 8, 12, and 14) was much lower compared with the rest of the tumours. Reduction of ROX/MNT in MB3, 5, 7, and 8 was accompanied by reduction of both UBE2G1 and 14-3-3γ, while MB14 had reduced expression of UBE2G1 only. In contrast, MB12 had "normal" levels of UBE2G1 and 14-3-3ε. It is important to note that the standard deviation for each data point did not exceed 18% of the mean value; however, as these data are presented in logarithmical form, incorporation of standard deviation in the graphs was impossible. These data raise the possibility that reduced expression of ROX/MNT

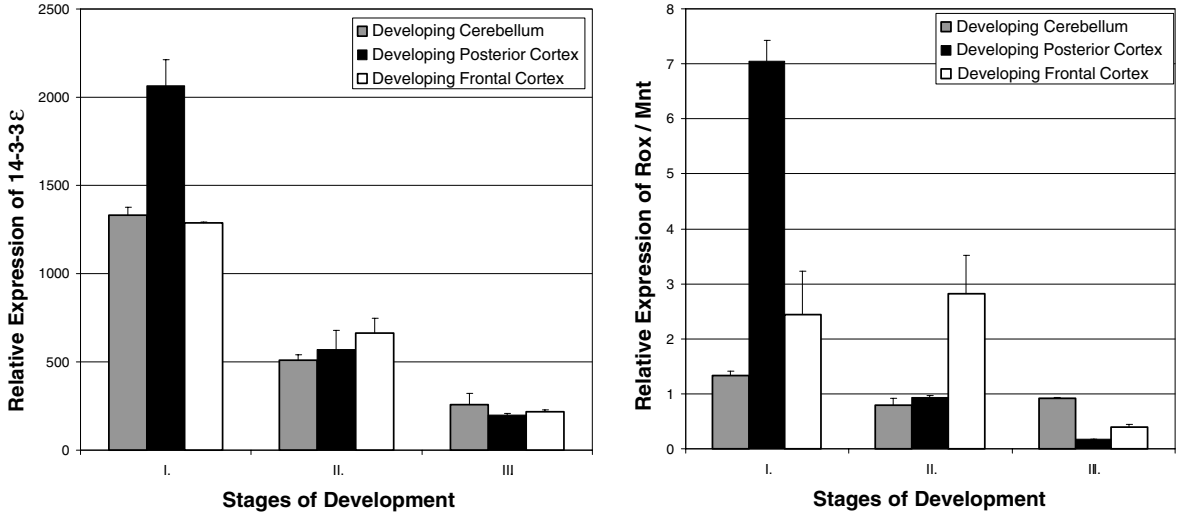

Fig. 2. Relative expression of ROX/MNT and 14-3-3ε in normal mouse brain. Stages of development were E14.5 (I.), D7 (II.), and D35 (III.). Developing cerebellum (grey), developing posterior (black) and frontal (white) cortex. The expression levels were normalised using HPRT1 as described in Section 2.4.

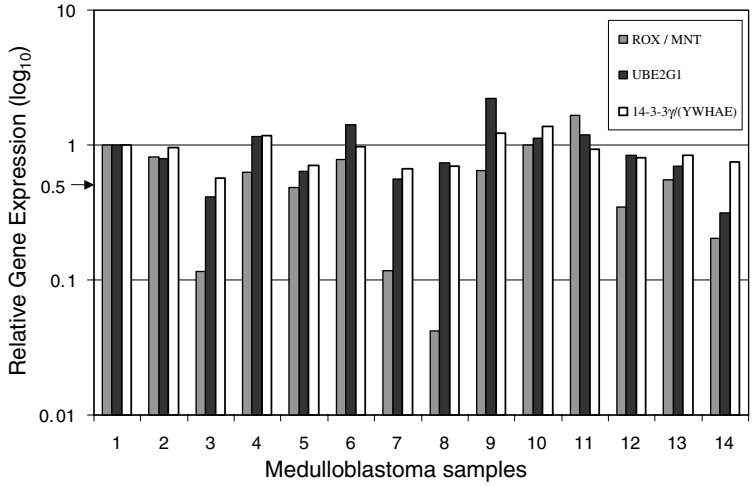

Fig. 3. Expression of ROX/MNT, UBE2G1 and 14-3-3ε in medulloblastomas. Relative expression of ROX (grey), UBE2G1 (black) and 14-3-3ε (white) in 14 medulloblastomas (1–14). The data were first normalised against the expression of ACTB, and then normalised against the expression level of MB1. The experiments were performed twice in triplicate. The expression levels were normalised using HPRT1 as described in Section 2.4.

(range of 2- to 20-fold reduction) in MB3, 5, 7, 8, 12, and 14, representing 6 of the 14 examined samples, is due to *ROX/MNT* haploinsufficiency. Deletion of a single allele of *ROX/MNT* may include deletion of *UBE2G1* and 14-3-3ε in MB3, 5, 7 and 8, and of *ROX/MNT* and *UBE2G1* in MB14.

3.4. c-MYC to ROX ratio of expression

A previous report had shown increased expression of oncogene c-MYC in medulloblastomas, which was associated with negative patient outcome [14]. ROX and c-MYC might form a complex and regulate their own expression using E-box (CANNTG) binding sites in their respective promoters [15]. Hence, we determined the relative ratios of c-MYC expression to ROX. The results (Fig. 4) showed significant upregulation of c-MYC in 4 of 14 medullobastomas, i.e. MB 6, 8, 9 and 12. In two of these cases (MB 8 and 12), ROX expression was reduced. Ratio of ROX to c-MYC in normal adult human cerebellum is also shown in Fig. 4.

4. Discussion

The goal of this project was to identify potential TSGs residing in 17p13.3, as predicted from the 35–50% of medulloblastomas in which 17p13.3 is deleted. Reduced amounts of the expression of one or more genes in RNA samples obtained from medulloblastoma tumours would suggest genetic deletion. In addition, expression of these genes in human and mouse cerebellum combined with their established function could pinpoint the disease-causing gene(s) enabling the development of a mouse model. Although cytogenetic data indicating haploinsufficiency in 17p13.3 have been available for some time [4,16,17], precise mapping of the deletion has not been achieved. The relatively small number of cases, even smaller number of preserved tumours, and lack of data on them have prevented the generation of a sufficient number of specific DNA probes to analyse the extent of the deletion in a given tumour. Identification of genes with reduced levels of expression is a powerful initial approach to assess allelic deletion and inactivation. Herein, we focused on a group of seven genes, 14-3-3ε, *HIC*−1, *ROX/MNT*, *KIAA*0399, *UBE2G*1, *ALOX*15, and *MINK* (see Fig. 1), which are located across the entire region of 17p13.3 and whose function is linked to tumorigenesis.

HIC-1, *ROX/MNT*, and *ALOX15* were previously proposed as potential TSGs [15–18]. *HIC-1* encodes a transcriptional repressor, expressed in tissues of mesenchymal and ectodermal origin; however, its expression pattern in neonatal and adult brain is not established [19]. *ROX/MNT* [15,20] encodes a member of the Myc/Max superfamily of transcriptional regulators involved in cancer ([for a Review, see [21]). Lipoxygenase gene, *ALOX15*, encodes an enzyme that converts arachidonic and linoleic acids into endogenous ligands for peroxisome proliferator–activated receptor-γ (*PPARγ*) regulating osteoblast differentiation [22]. In addition, expression of ALOX15 is downregulated in colorectal cancer [18]. 14-3-3ε encodes a signal transduction protein, which was discovered to be homozygously deleted in lung cancer. Reintroduction of the gene prevented tumour cell growth by blocking the cells from leaving the G_2 phase [23], and haploinsufficiency of this gene is associated with neurogenic tumours [24]. *MINK* encodes a GCK protein kinase that is upregulated during murine cerebral differentiation, raising the possibility that it may play a general role in terminal differentiation in other tissues [25]. Although there is very little information

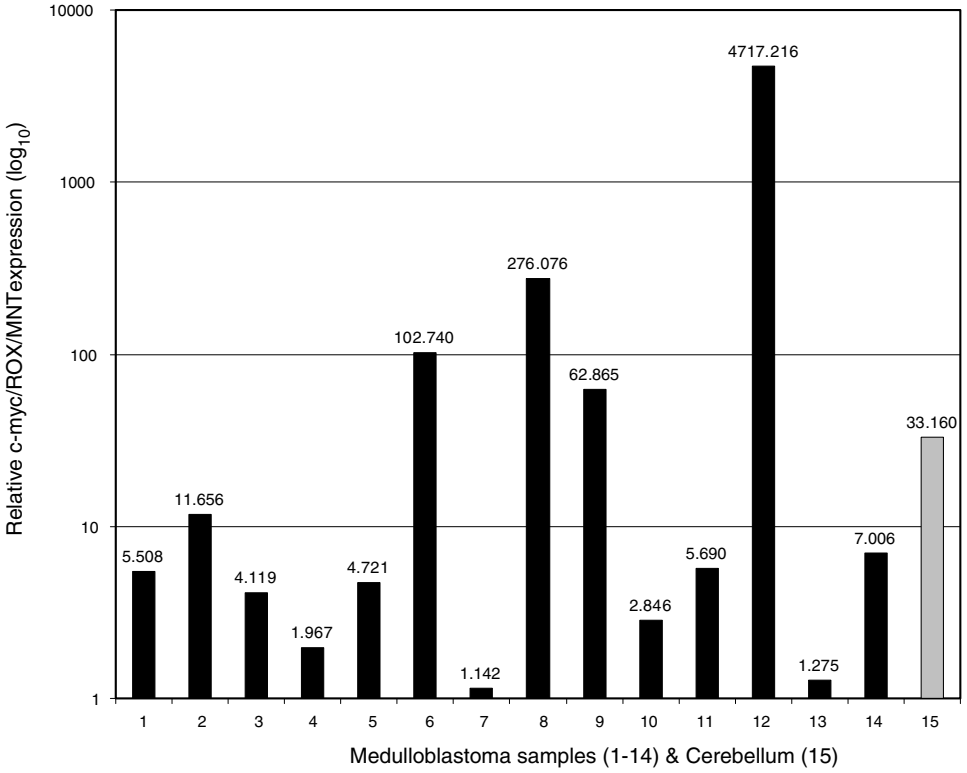

Fig. 4. Ratio of expression between c-MYC and ROX/MNT in medulloblastomas and normal adult cerebellum. Ratio of expression of c-MYC compared with ROX in 14 medulloblastomas are shown in log10 scale (solid bars). Expression in normal adult human brain is shown in grey.

on ubiquitin-protein ligase G1 (UBE2G1), other members of this family of ubiquitin-conjugating enzymes regulate cell proliferation [26]. Expression of KIAA0399 in the brain combined with the presence of a structural motif, the chromodomain [for a Review, see [27]], found in many chromatin-associated proteins, suggests the possibility of a regulatory function for this protein.

Of 119 genes in 17p13.3, previous studies have predicted four of these-*HIC1*, *OVCA2 ROX/MNT*, and *ALOX15*- as candidate TSGs in cancers other than medulloblastoma. *OVCA2* [for a Review, see [28]] was not considered further, as the Unigene database contained no data supporting its possible expression in human, mouse, and rat tissues of neuronal origin. None of the remaining 115 genes appeared to be candidate TSGs, based on our selection criteria. However, we examined the transcript levels of *14-3-3ε*, *KIAA0399*, *UBE2G1*, and *MINK*, because of the participation of these genes in cellular processes potentially related to cancer.

We found that ROX/MNT expression was reduced in 6 of 14 analysed samples (Fig. 3). ROX/MNT is expressed both in the mouse embryonic hindbrain, and in neonatal and postnatal cerebellum (see Fig. 2). Its expression appears to be only slightly reduced in postnatal tissues compared with that found in the hindbrain/cerebellum precursor, suggesting a specific function in the maintenance of cerebellar cells. The reduced expression of ROX/MNT in medulloblastomas could be caused by at least three factors: deletion of one allele of *ROX/MNT* in tumour cells, structural and/or epigenetic changes in regulatory regions of the *ROX/MNT* locus, or as a consequence of large chromosomal abnormalities in cancer cells leading to the deregulation of a battery of genes [2,5,6,8–10]. The present expression data are consistent with the idea that *ROX/MNT* is deleted from 17p13.3 in 35–50% cases of medulloblastoma. Loss of heterozygosity of both *ROX/MNT* [17] and *HIC-1* [16] has been reported. Thirty-six medulloblastomas showed a loss of heterozygosity of *ROX/MNT* gene [17], while *HIC-1* [19,29] which encodes a transcriptional repressor not expressed in cerebellum [19], was haploinsufficient in 15 (42%) of 36 medulloblastomas [16]. Since *ROX/MNT* and *HIC-1* are located in relative proximity (Fig. 1), this finding directly supports our prediction that *ROX/MNT* lies within the deleted region. In addition, it is possible that ROX/MNT could be deregulated in medulloblastomas containing an intact *ROX/MNT* locus. It has been suggested that epigenetic silencing of the *HIC-1* locus could contribute to the pathogenesis of medulloblastomas [30]. The human neurotropic polyomavirus, JCV, was found to be integrated in 77% of genomic DNAs obtained from 43 well-characterised medulloblastomas [31]. However, it is not known

whether some of the integration sites were in the 17p13.3 region.

An earlier study examined expression of ROX/MNT in 36 human medulloblastomas using simple RT-PCR analysis [17]. After 33–35 PCR cycles, expression of ROX/MNT was found in all medulloblastomas. In addition, a specific, albeit weak, MNT/MAX protein-DNA complex with a high affinity Myc/Max binding site was detected using electrophoretic mobility shift assays in six tested cellular extracts obtained from the medulloblastomas. There is no discrepancy between the earlier findings [17] as our present data also show expression of ROX/MNT in the tumour samples. However, quantitative analysis performed here allowed comparisons between individual medulloblastomas. Moreover, the earlier study demonstrated loss of heterozygosity of the *MNT* locus at the genomic DNA level obtained from their tumours [17], supporting our results.

A poor patient outcome in MB was associated with high levels of c-MYC expression [14], a gene regulated by the MAX/MAD superfamily found on human chromosome 8. These levels were a consequence of *c-MYC* gene amplification in 37.5% of cases [14]. Here, we found four cases of increased c-MYC expression over ROX expression, including two tumours in which ROX expression was considerably diminished (Fig. 4). Reduced expression of ROX may disrupt the balance between its partners, including c-MYC, N-MYC, MAX, MADs, and MXI1 [21].

Of the 14 tumours studied, three of them originated from patients who died of their cancer. All three patients had a reduction of ROX expression by at least 50%. Only one patient died who had elevated c-MYC expression. Since in each instance death correlated with deregulation of ROX, ROX should be considered to play a crucial role in clinical outcome. The remaining eleven patients survived.

In summary, we provide direct evidence that ROX/MNT transcript levels are significantly reduced (in the range of between 2- to 20-fold decrease), but not abolished in 6 of 14 medulloblastomas. Increased expression of c-MYC was observed in two of these medulloblastomas. This information will aid cytogenetic studies to determine if a simultaneous deletion of genomic DNA harboring *ROX* and *HIC1* exists. Another important step to characterise this cancer will be to spatially characterise the expression of ROX/MNT and other deregulated genes in the tumour samples. The methodology used here, in combination with laser capture microdissection, appears to provide better quantitative answers than data derived from non-quantitative *in situ* hybridisations. Molecular studies of transcriptional repression by ROX/MNT in cell lines derived from medulloblastoma, as well as targeted deletion of ROX/MNT, from the mouse genome, will provide more definitive answers as to whether haploinsufficiency of ROX/MNT is a direct cause of this devastating form of cancer [20]. In addition, haploinsufficiency of Patched 1, a receptor in sonic hedgehog signalling, a gene not found in 17p13.3, is also associated with MB formation [32]. Availability of mouse models of targeted deletion of Patched 1 [32,33], ROX/MNT [20], and Bmi1 [10] will greatly aid the identification of processes regulated by these genes in conjunction with oncogenesis [34].

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