Introduction

The human placenta, a globally hypomethylated tissue, is becoming increasingly known for harbouring unmethylated repetitive sequences [1]. The epigenetic activation of repeat sequences in the placenta is demonstrated by the finding that many repetitive elements that are normally silenced by methylation in somatic tissues are transcribed in the placenta [1–5]. Of the repeat elements, the Alu elements, long interspersed nuclear elements (LINEs), and satellite regions display a reduced level of methylation in the placenta compared to the majority of somatic tissues [6–8]. The unique placental expression of these normally silenced repeat elements suggests a functional role for these sequences in the placenta, a role exemplified by the retrovirus-derived repeat elements (LINEs), which is essential for trophoblast differentiation during placental development [9,10].

The loss of DNA methylation at certain repetitive sequences has been shown to be a hallmark in some cancers [11,12]. Hypomethylation of repetitive elements has been associated with genome instability, a trait that is commonly associated with cancer [12–14]. The parallel hypomethylation of repetitive elements in the placenta and in cancer is just one of the many well-documented similarities between placental and cancer cells. These two cell types also share common molecular mechanisms that are thought to regulate their similar proliferative, migratory and invasive phenotypes [15]. The invasion of the trophoblast during placental development is noted to be similar to tumour invasion in cancer [16,17]. In addition, the co-existence of genetically dissimilar fetal and maternal cells in the placenta echoes the interface of genetically normal and genetically abnormal cancer cells that occurs in neoplasms.

We previously identified a novel retrotransposon-derived transcript of the KCNH5 gene that is hypomethylated and expressed specifically in the human placenta [18]. The expression of this placental transcript (referred to in this study as pKCNH5; previously called transcript 3a) is regulated by a hypomethylated promoter and first exon region that recently arose from a SINE (Alu) retrotransposition event. This region is silenced and methylated in all studied non-placental tissues. Data from the Roadmap Epigenomics Project (http://www.roadmapepigenomics.org/) show that placenta is the only normal tissue with DNAseI hypersensitivity at the pKCNH5 promoter, supporting its unique hypomethylated status [19]. From this same source, MeDIP data confirms methylation of the pKCNH5 promoter in somatic tissues.

Of the six reported human mRNA sequences for KCNH5 in GenBank (http://www.ncbi.nlm.nih.gov/genbank/), two sequences begin translation using this upstream SINE-derived placental promoter, giving rise to two pKCNH5 transcripts that differ in their 3’ ends [20]. The remaining four mRNA sequences in GenBank are translated using a downstream promoter, which is unmethylated and used for expression in a wide range of somatic tissues [10]. This downstream promoter gives rise to somatic KCNH5 transcripts that also differ in their 3’ ends (collectively referred to in this study as sKCNH5; previously called transcript 1b). Although “pKCNH5” and “sKCNH5” are not the current nomenclature for the reported KCNH5 transcripts, these terms are being used in this study to highlight whether the placental (p) or somatic (s) promoter is being used for KCNH5 transcription. Based
on the reported transcripts, each promoter of KCNH5 is predicted to give rise to two protein isoforms, resulting in four predicted isoforms for KCNH5. Unexpectedly, one pKCNH5 mRNA and two EST sequences in Genbank were derived from melanotic melanoma (BC043409, BQ439615 and BX201705).

The KCNH5 gene (also known as EAG2), encodes an EAG voltage-gated potassium channel that is involved in the regulation of cell cycle and proliferation [21]. Previous work investigating the oncogenic potential of a closely related EAG potassium channel (EAG1/KCNH1) showed homologous to KCNH5 [22,23] detected expression in several somatic cancer cell lines (including melanoma), normal adult brain and placenta, but not in other normal somatic cells [21]. These expression patterns prompted investigation of KCNH5 as a potential cancer biomarker [24]. Although the function of KCNH1 is better understood than that of KCNH5, both of these genes are thought to be involved in cell cycle regulation and tumour progression in cancer [21,25].

Given that the placental-specific transcript of KCNH5 has been detected in melanoma [26,27], we aimed to investigate whether its retrotransposon-derived promoter, which is hypermethylated in those studied normal tissues from the body [18], becomes hypomethylated in melanoma. We hypothesised that genes that are hypomethylated and active in the placenta may become inappropriately activated in cancer tissues by similar epigenetic mechanisms.

Materials and Methods

Ethics Statement

The samples of human placenta were obtained with written consent under the approval of the Lower South Regional Ethics Committee (New Zealand). The human fetal tissues (brain, liver, heart, stomach, adrenal) were obtained with written consent under the approval of the Otago Ethics Committee (New Zealand). The human adult somatic tissues (brain, kidney, heart, liver, spleen, pancreas, lung and colon) were obtained at autopsy, following informed written consent, under the provisions of Section 3 of the Human Tissue Act 1964 [28]. The New Zealand melanoma (NZM) cell lines used in this study were generated from surgical samples of metastatic melanoma, obtained with written consent from all patients under the guidelines and specific approval of the Auckland Area Health Board Ethics Committee (New Zealand).

Sample Collection

The 25 human melanoma (NZM) cell lines used in this study were generated from American Joint Committee on Cancer (AJCC) Stage III or IV tumours of consenting patients as described previously [29–31]. Phenotype data was available for ten of the melanoma cell lines; categorised as either ‘more invasive’ or ‘less invasive’ (previously referred to as Motif 1 and Motif 2 [32]). The more invasive cells (n = 5) are NZM09, NZM11, NZM22, NZM40, NZM52 and the less invasive cells (n = 5) are cell lines NZM06, NZM12, NZM15, NMZ42, NMZ45. In addition to the 25 melanoma cell lines, RNA and DNA from three patients’ metastatic tumours were included in this study (samples NZM53T, NZM58T, and NZM62T). Two of the metastatic tumour samples (NZM53T and NZM58T) also had derivative cell lines in this study (NZM53 and NZM58).

The samples of human placenta consisted of 31 first-trimester placentas and one term placenta. The 31 first-trimester human placenta samples were collected and processed as described previously [18]. Six first-trimester placenta samples were of 35–41 days gestation, 12 samples were of 42–48 days gestation, and 13 samples were of 49–55 days gestation. The three pooled sections from one human term placenta (maternal facing, middle and fetal facing) were processed as described previously [18]. The human fetal tissues (brain, liver, heart, stomach, adrenal) were previously collected from medically terminated pregnancy tissues. The adult somatic tissues (brain, kidney, heart, liver, spleen, pancreas, lung and colon) were collected at autopsy from consenting patients on the basis that the tissues were non-diseased (as per medical records). Peripheral blood was collected from healthy donors.

Gene Expression Analysis

RNA was extracted from melanoma cell lines and tumour tissue using a combined Trizol/column clean-up protocol as described in [24]. For non-melanoma samples, RNA was extracted from 50 mg of frozen tissue (placenta, fetal or adult tissues) using the PureLink Micro-to-Midi Total RNA Purification System (Invitrogen, Cat #12183-018). Complementary DNA was made from 300 ng RNA by using the Applied Biosystems High Capacity RNA-to-cDNA Kit (Applied Biosystems, Cat #4378406) following the manufacturer’s protocol. To test cDNA quality, a Reverse-Transcriptase-PCR (RT-PCR) was performed on all samples for the beta-2-microglobulin (B2M) housekeeping gene. Melanoma samples were then screened for KCNH5 expression using the RT-PCR protocol described previously [18]. Primer sequences for RT-PCRs are listed in Table S1. The presence of a 425 bp product confirmed expression using the pKCNH5 promoter and the presence of a 369 bp amplicon confirmed expression using the sKCNH5 promoter. RT-negative and no-template (water) controls were used in PCRs to exclude non-specific amplification and genomic contamination. PCR product was then purified from all samples in both pKCNH5 and pKCNH5 RT-PCRs using QIAquick PCR purification kit (Qiagen, Cat #28106) following the manufacturer’s protocol. The purified PCR product was sequenced using the forward (transcript-specific) primers by the Genetic Analysis Services (Department of Anatomy, University of Otago) to determine which KCNH5 promoter was used for transcription in each melanoma sample. Sequences were aligned to the reported pKCNH5 and sKCNH5 sequences [33] and alignment within the first 114 bp of pKCNH5 and 61 bp of sKCNH5 confirmed the specific gene product. Beyond this point the sequence of the two transcripts is the same (and continues until exon 7).

Quantitative Gene Expression Analysis

Primers for RT-qPCR were designed to the promoter and unique exons 1 and 2 of pKCNH5 using the IDT RealTime PCR Custom Assay Design online tool (http://eu.idtdna.com/Scitools/Applications/RealTimePCR). Primer sequences are listed in Table S2. Transcript abundance was measured using Power SYBR® Green PCR Master Mix (Applied Biosystems, Cat #4367639) with ROX reference dye on an ABI 7300 Real-Time PCR System. Standard curves for pKCNH5 and two reference genes, GNB2L1 and RPL13A were generated to assess primer efficiency. Relative transcript abundance was calculated by using qBase+ software [34] with target-specific amplification efficiency correction and normalisation to the GNB2L1 and RPL13A reference genes. For each assay, reactions were performed in triplicate and Ct values were excluded if they differed by more than 0.55 Ct. Relative transcript abundance of pKCNH5 was scaled to the sample with the lowest transcript abundance (NZM61).

Sequenom MassARRAY EpiTYPER Analysis

DNA was extracted from melanoma cell lines and tumour tissue by using a Genomic DNA Isolation Kit (Norgen Biotech Cat.
Melanoma Exhibits Placental Epigenetic Features

Gene Expression Analysis of pKCNH5 in Melanoma

KCNH5 expression was examined by RT-PCR to determine whether melanoma samples used the placental promoter for KCNH5 transcription. In the pKCNH5 RT-PCR, the presence of a 423 bp PCR product was detected in 11 of the 25 melanoma samples (Figure 1B). Sequencing all samples from this PCR confirmed the use of the placental promoter and express pKCNH5, as well as the positive control sample of placenta. C. Screening cDNA for the internal control B2M gene (109 bp product). The varying product intensities are likely to result from differences in B2M mRNA template produced by each melanoma cell line. Sample NZM15 consistently displayed negligible B2M expression yet was positive for pKCNH5 expression.

Methylation Analysis of pKCNH5 in Melanoma

Sequenom analysis was used to quantify the level of retrotransposon methylation within the promoter region of pKCNH5.
Mean pKCNH5 methylation was lower in melanoma samples that expressed the placental transcript (25%) and higher in melanoma samples that did not express the transcript (50%) (Figure 2B). The low levels of methylation in first trimester placenta (11%) and term placenta (15%) and high levels of methylation in pooled somatic tissues (89%) and pooled fetal tissues (93%) are consistent with our previously published results [18] (which are included in Figure 2).

Quantitative Expression Analysis of pKCNH5 in Melanoma

Quantitative expression analysis of pKCNH5 in melanoma showed an inverse relationship between pKCNH5 expression and promoter methylation (Figure 3). Not all hypomethylated tumour samples showed expression of pKCNH5, but there appears to be a threshold at which pKCNH5 hypomethylation may become permissive of expression, i.e., less than approximately 50% methylation. Indeed, pKCNH5 transcript expression in cell lines with above 50% promoter methylation was significantly lower than those with below 50% \( (p = 0.0017; \) Figure 4). Additional analysis of individual CpG sites in the pKCNH5 promoter did not reveal a specific CpG site whose methylation was more predictive of expression than that of the promoter as a whole.

Gene Expression and Methylation Analysis of sKCNH5 in Melanoma

Our previous comparison of placental and somatic tissues suggested that transcription of pKCNH5 is independent of that from the sKCNH5 promoter. For sKCNH5, RT-PCR followed by sequencing revealed that the somatic promoter was expressed in five of the 25 melanoma cell lines (NZM01, NZM06, NZM12, NZM15, NZM23, NZM53, NZM58, NZM52, NZM53T and NZM58T) and 17 samples did not express pKCNH5. Error bars represent the 95% confidence interval of the mean.

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(Figure 2A). Mean pKCNH5 methylation was lower in melanoma samples that expressed the placental transcript (25%) and higher in melanoma samples that did not express the transcript (50%) (Figure 2B). The low levels of methylation in first trimester placenta (11%) and term placenta (15%) and high levels of methylation in pooled somatic tissues (89%) and pooled fetal tissues (93%) are consistent with our previously published results [18] (which are included in Figure 2).
and sKCNH5 promoters in melanoma samples (Figure S3B). The association between sKCNH5 methylation and sKCNH5 expression in melanoma is shown in Figure S3C.

Discussion

The EAG class of ion channels is overexpressed in many cancers [35,36]. It is suggested that they regulate cell proliferation through control of the entry of cells into the G1 phase of the cell cycle [37,38]. Therefore, molecular changes to EAG channels may result in dysregulation of cellular proliferation, one of the hallmarks of carcinogenesis. In this study, the expression of pKCNH5 in melanoma cells correlates with hypomethylation of the promoter region, suggesting that this epigenetic change may accompany the expression of pKCNH5 which contributes to the oncogenic change in melanocytes [39,40].

DNA hypomethylation in melanoma has been associated with aberrant gene expression and the unsilencing of normally methylated repeat regions. Studies have shown that hypomethylation in melanoma correlates with activation and expression of some of the cancer testis (CT) genes [41,42]. CT genes encode protein antigens that are normally expressed in adult testicular germ cells (and not in somatic tissues) [43,44]. Given the number of hypermethylated (and aberrantly activated) CT genes in melanoma, they are now being studied as potential targets for slowing melanoma progression [42,44]. In addition, hypomethylation of the non-coding retrotransposon sequence, long interspersed nuclear element-1 (LINE1), was identified as a feature of melanoma, with the degree of LINE1 hypomethylation positively correlated with melanoma progression [45].

In this study, the expression of pKCNH5 is significantly higher in melanoma cell lines that have lower levels of promoter methylation. Hypomethylation alone does not guarantee increased gene expression, but can act permissively to allow gene expression. There were no differences in promoter methylation or pKCNH5 expression between the “more invasive” and “less invasive” melanoma cell lines (data not shown). This suggests that pKCNH5 is not involved in invasion, which was not surprising given that KCNH5 is thought to be a regulator of cell proliferation instead of invasion [37,38]. Although the use of cultured melanoma cell lines presents a risk for culture-induced epigenetic changes, the small differences in methylation (12% and 13%) between the tumour samples (NZM53T and NZM58T) and their derivative cell lines (NZM53 and NZM58) suggests that the cell lines did not acquire significant epigenetic aberrations in culture.

Similar to our previous findings [18], methylation of the somatic KCNH5 promoter (sKCNH5) was low in all of the examined tissues. Expression of sKCNH5, the promoter of which is not derived from a retrotransposon, is not associated with pKCNH5 expression in melanoma samples (Figure S3A). The methylation of sKCNH5 is also not related to pKCNH5 methylation in melanoma (Figure S3B). Importantly, we and others have found that primary adult melanocytes do not express either transcript of KCNH5 (Supplementary Figure 4 in [18] and data from the UCSC browser [26]). According to data from the Roadmap Epigenomics Project, melanocytes show no DNase1 sensitivity at the sKCNH5 promoter, suggesting a condensed chromatin state (not transcriptionally

Figure 3. Mean promoter methylation and relative expression of pKCNH5 in melanoma cell lines. Average promoter methylation (%) is depicted on the left-hand axis and displayed as blue columns. pKCNH5 expression (relative to the lowest expressing cell line; NZM61) is depicted on the right-hand axis and shown as red circles. Error bars represent the standard error of the mean. Samples labeled “(T)” refer to samples from metastatic tumour RNA/DNA (NZM53T, NZM58T and NZM62T).

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active) at that site [19]. Thus the finding that melanoma samples express the placental transcript of KCNH5 is notable given that healthy (non-cancerous) melanocytes do not express either transcript of this gene.

We have previously demonstrated that the retrotransposon-derived transcript of KCNH5 is expressed exclusively in the human placenta. We detected high levels of methylation in its promoter and did not detect expression in any of the somatic tissues (brain, kidney, heart, liver and spleen) [18]. Therefore, the expression of KCNH5 and its coordinate hypomethylation in melanoma may reflect a tumour-associated epigenetic change. Hypomethylation and expression of many retrotransposon-derived sequences in the placenta is assumed to contribute to the invasive features of the primate hemochorial placenta [46,47]. We speculate that hypomethylation in melanoma cells may involve mechanisms similar to those in the placenta and contribute to their malignant phenotype. Analysis of other retrotransposon-derived promoters in melanoma may provide further insight into hypomethylation as an event that is part of the cancer transformation pathway.

Supporting Information

Figure S1 End-point RT-PCR screening for sKCNH5 in melanoma. A. Detection of sKCNH5 product by agarose gel electrophoresis. All samples were sequenced and red asterisks indicate the five melanoma samples that were confirmed to use the somatic promoter (NZM01, NZM06, NZM40, NZM52, NZM53) and express sKCNH5 (369 bp). B. RT-negative samples confirm specificity of the sKCNH5 RT-PCR.

Figure S2 Mean promoter methylation for sKCNH5. A. Genomic map of sKCNH5 amplicon that was examined by Sequenom. Coordinates refer to the genomic location with respect to the sKCNH5 transcription start site. Red circles represent individual CpG sites. Black arrows represent primers used to amplify the 445 bp product, which contained 38 CpG sites that were analysed for methylation. B. Columns represent mean CpG methylation for the amplicon. Solid bars represent Sequenom data from the present study and lined bars represent previously published Sequenom data [18]. Error bars represent the 95% confidence interval.

Figure S3 Relative pKCNH5 expression compared to presence or absence of sKCNH5 expression in melanoma. A. Expression of pKCNH5 was not related to expression of sKCNH5. Data points represent relative pKCNH5 expression values (from qRT-PCR) for the 25 melanoma cell lines, grouped by presence or absence of sKCNH5 expression. An unpaired T-test with Welch’s correction was performed, yielding a p-value of 0.384. B. Methylation of pKCNH5 was not related to sKCNH5 expression. Data points represent pKCNH5 and sKCNH5 methylation values for the 25 melanoma cell lines. A linear regression analysis was performed, yielding a p-value of 0.335. C. The relationship between sKCNH5 expression and methylation. Data points represent sKCNH5 methylation and expression values, grouped by presence or absence of sKCNH5 expression (based on sequencing of end-point RT-PCR product). An unpaired T-test with Welch’s correction was performed, yielding a borderline-significant p-value of 0.029, which is largely attributable to a single outlying point (a non-expressing sample with 60% methylation).

Table S1 Primer sequences for end-point RT-PCR gene expression analysis of pKCNH5 and sKCNH5 in melanoma cell lines.

Table S2 Primer sequences for quantitative RT-PCR gene expression analysis of pKCNH5 in melanoma cell lines.

Table S3 Primer sequences for Sequenom promoter methylation analysis of pKCNH5 and sKCNH5 in melanoma cell lines.

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Author Contributions

Conceived and designed the experiments: ECM IMM. Performed the experiments: ECM HER XC. Analyzed the data: ECM HER XC. Contributed reagents/materials/analysis tools: ARJ BCB IMM. Wrote the paper: ECM HER XC.
