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Frequent hypomethylation of *PTGS2* gene promoter in human term placenta

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Summary

Background: Gene expression profiles of several tumor suppressor genes are regulated by the methylation and demethylation of their promoters. Here, we aim to identify and quantify the methylation status of four tumor suppressor genes from placentas at term and compare them with the maternal white-blood-cells.
Methods: In order to achieve this objective, DNA enriched from twenty placentas at term and maternal white blood cells was bisulfite-converted and amplified using quantitative real-time methyl-light polymerase chain reaction for the four-genes studied (*RASSF1A, APC, RAR-beta, and PTGS2*).
Results: Among the four genes examined, *RASSF1A, APC* and *RAR-beta* promoter regions were hypermethylated in all the placental samples compared with maternal WBCs. Strikingly, *PTGS2* was found to be hypomethylated in the placentas compared to the maternal cells.
Conclusion: Since placental DNA represents fetal methylation profile and it is an established fact that there is certain amount of cell free circulating DNA in human plasma/serum, these data strongly suggest that hypermethylation of *RASSF1A, APC and RAR-beta* can be used as gender independent biomarkers to distinctly identify placental DNA in maternal blood. In-addition, this is the first report which demonstrates hypomethylation of *PTGS2* locus which may have important clinical implications e.g. placental abnormalities.

Key words

DNA methylation; tumor suppressor genes; human term placenta; gene expression.

Introduction

DNA methylation patterns change during initiation and progression of cancer, leading to alterations in the gene expression profiles of a variety of relevant genes, e.g., those involved in regulating cellular proliferation, invasion and migration (Esteller, 2007). There is striking similarity between placental development and tumor growth. Thus in order to assess the role of DNA methylation in normal and abnormal placental development the focus has been on genes related to development of tumors.

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One of the earliest investigations to examine the methylation status of some candidate tumor suppressor genes in the placenta was performed by Chiu et al. (2007). They discovered hypermethylation of the *RASSF1A* gene in term placentas, whereas this gene target was not hypermethylated in any maternal white blood cell (WBC) samples. Hypermethylation of gene promoters leads to changes in gene expression and may contribute to placental pathologies (e.g., preeclampsia). Herein we have identified and quantified the methylation status of four tumour suppressor genes from placentas at term and compare them with the maternal WBCs.

Materials and Methods

Sample collection

Placental and peripheral blood samples were collected from 20 women admitted for delivery at the Aga Khan University Hospital for uncomplicated pregnancies at gestational age > 37 weeks. Peripheral blood samples were collected before the onset of labor from the same women from whom placental samples were acquired. The study and the method for collecting human clinical samples were approved by the Aga Khan University Ethics Review Committee. Written informed consent was obtained from all the participants prior to collection of the samples.

DNA extraction

DNA was extracted using Tissue DNA extraction kit (Qiagen, Maiz, Germany) from 1 cm^3 piece of placental tissue dissected from the fetal surface, near the umbilical cord. Concurrently DNA was also extracted from a 5 ml maternal blood sample using QIAamp® Blood Mini Kit (Qiagen, Mainz, Germany) according to the "blood and body fluid protocol". A totally methylated DNA served as a positive control.

Bisulphite modification and quantitative-methylation specific real-time PCR

3 μg of extracted DNA from the placenta and the leukocytes was bisulfite converted using the Methyl Easy *Xceed* DNA Modification Kit (Human Genetic Signatures Pty, Sydney, Australia). A bisulphite converted totally methylated DNA was used as a positive control. Real-time PCR was established for four genes, namely, *APC, RASSF1A, RAR-β and PTGS2*.

β-ACTIN was used as an internal control to detect the presence of total DNA. Real-time quantitative PCR analysis was performed using Chromo4 Real-time PCR Detector (Bio-Rad, Hercules, CA). All experiments were performed in a reaction volume of 25 μl with 2 μl bisulphite converted DNA with the following reaction conditions: 1X Go flexi buffer, 5.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 450 nM of forward and 450 nM of reverse primers, 125 nM of TaqMan probe and 1 U of Go Flexi *Taq* Polymerase (all from Promega, Fitchburg, WI). Cycling conditions were 5 min initial denaturation at 95 °C, 55 cycles of 15 s denaturation at 95 °C, 1 min annealing and extension at 60 °C and fluorescence detection at the end of each cycle. Methylated DNA served a positive control. Blank reaction with water substituted for DNA

Table 1 – Primers and Probe Sequences.

Gene symbol	5′ to 3′ Forward primer	5′ to 3′ Taqman probe	5′ to 3′ Reverse primer
RASSF1A	GCGTTGAAGTCGGGG TTC	FAM-ACAAACGCGAA CCGAACGAAACCA-BHQ1	CCCGTACTTCGCTAACT TTAAACG
PTGS2	CGGAAGCGTTCGGG TAAAG	FAM-TTTCCGCCAAAT ATCTTTTCTTCTTCGCA-BHQ1	AATTCCACCGCCCCAA AC
RAR-β	AGAACGCGAGCGATT CGAGTAG	FAM-ATCCTACCCCG ACG ATACCCAAAC-BHQ1	TACAAAAAAACCTTCC GAATACGTT
APC	TTATATGTCGGTTACGT GCGTTTATAT	FAM-CCCGTCGAAAAC-CCGCCGATTA-BHQ1	GAACCAAAACGCTCCC CAT
ACTB	TGGTGATGGAGGAGGT TTAGTAAGT	FAM-ACCACCAC-CCAACACACAATA ACAAACACA-BHQ1	AACCAATAAAACCTAC TCCTCCCTTAA

served as negative control. A five-point standard curve for each gene was prepared using serial dilutions of 3 μg of bisulfite converted methylated DNA. All primers and probes were obtained from Biosearch Technologies (Novato, CA). Primer and probe sequences used were previously described by (Eads et al., 2001; Fackler et al., 2004; Yegnasubramanian et al., 2004) and presented in Table 1.

Statistical analysis

To quantify the relative proportion of methylation at the candidate gene loci in all the samples (placental and maternal), a methylation index was determined for each gene in each tissue sample. The methylation index was defined as the ratio of the normalized (normalized to positive control, totally methylated DNA) amount of methylated templates at the promoter region of interest to the normalized amounts of converted beta actin (*ACTB*) templates in a given sample. These *ACTB* template do not have CpG sequences so they are unaffected by bisulphite conversion.

In order to assess the statistical significance in the methylation status of each gene locus between maternal WBC and placental samples, non-parametric Mann-Whitney test was employed. All analyses were conducted using SPSS (Chicago, IL) software Version 16.0.

Results

In this report we have compared the methylation status of four tumour suppressor genes (*RASSF1A, APC, RAR*-β *and PTGS2*) in 20 maternal WBC samples with 20 placental samples obtained from the same patients using methyl-light technology. In order to quantify the methylation status at each gene locus a standard curve

Table 2 – Cycle threshold (†) values in the placenta (n=20) and maternal cells (n=20).

Genes	Placenta			Maternal Cells			Methylated DNA*
	Mean	St.dev	Range	Mean	St.dev	Range	Mean
Actin	23.3	1.1	4.9	23.6	1.4	0.00	22.6
RASSF1A	22.6	3.3	12.9	0.00	0.00	0.00	22.5
APC	24.7	1.0	3.7	0.00	0.00	0.00	21.9
RAR-β	23.7	2.1	7.2	30.7	1.8	0.00	23.6
PTGS2	32.7	2.0	7.5	29.7	2.2	0.00	23.7

*Methylated DNA is artificially converted totally methylated DNA.
† Cycle threshold is the cycle number at which a sufficient number of copies has been made from template DNA while performing a polymerase chain reaction (PCR). In quantitative PCR (Q-PCR) this cycle number is related with enough fluorescence to be detected.

Table 3 – Methylation Index (MI).

Genes	MI values in placenta	MI values in maternal leukocytes
RASSF1A	1.0	0.00
APC	0.2	0.00
RAR-β	1.0	0.01
PTGS2	0.003	0.03

The MI was standardized based on the gene concentration found in the methylated DNA (=1.0).

was developed using dilutions of methylated DNA. The r^2 value for all the standard-curves was -0.99.

The results are summarized in Tables 2 and 3. *RASSF1A* and *APC* were found to be hypermethylated in all the placental samples while none of the maternal WBC samples showed methylation at these gene loci. *RAR-β* was found to be partially methylated in the placenta and maternal samples. However, the level of methylation was considered to be significantly higher ($p<0.001$) in placental samples compared to maternal WBC controls as shown by methylation index values. *PTGS2* gene locus also showed differential methylation levels in term placentas and maternal WBC samples; however this locus had a significantly lower methylation in the placental derived DNA as compared to maternal WBC samples.

Discussion

Differences in the DNA methylation profiles of maternal and fetal DNA has made detection of cell free circulating DNA in the maternal blood a powerful non-invasive method for prenatal diagnosis (Nygren et al., 2010). Although the hypermethylation of *RASSF1A, APC and RAR-beta* in human placenta has been reported previously

(Chiu et al., 2007; Novakovic et al., 2008), since DNA methylomes are population specific the current study describes for the first time these changes in Pakistani population.

DNA methylation plays an important role in placental development as evidenced from studies using the demethylating agent 5-Aza-2-deoxycytosine (5-deoxy-Aza). Administration of 5-deoxy-Aza to pregnant rats during different stages of placental development disrupted trophoblast proliferation (Serman et al., 2007; Vlahovic et al., 1999). Further, 5-deoxy-Aza inhibited migration of choriocarcinoma cell lines (Rahnama et al., 2006), suggesting a role of DNA methylation in migration and invasion of trophoblast cells.

Placenta share many similarities with cancer such as trophoblast proliferation and migration and neo-vascularization. In this context, it has been shown recently that the promoters of several tumour suppressor genes get hypermethylated in placenta including those of *RASSF1A*, *APC* and *Maspin* (Chiu et al., 2007; Dokras et al., 2006; Wong et al., 2008). Hypermethylation of *RASSF1A* and *APC* noted in our study is consistent with previous studies. Further, we noted hypermethylation of *RAR-β* gene which is a repressor of Wnt signaling pathway. A previous study has shown that four genes which negatively regulate Wnt signaling pathway are hypermethylated in term placentas (Novakovic et al., 2008).

Herein, we are reporting for the first time hypomethylation of the gene locus *PTGS2* (Cox2). This finding might have important implications to placental abnormalities like preeclampsia. A previous report indicated that *PTGS2* expression increases in placentas derived from pre-eclamptic women and is correlated with increased levels of Cox2 products, prostaglandin and thromboxane-A2. Further, that study showed that there was infiltration of leukocytes in vascular smooth muscle and vasoconstriction presumably contributing to preeclampsia (Walsh and Shah, 2007).

One of the major problem faced by many investigators is this area is to correlate the methylation status of the gene loci with the expression profile of these genes. There is significant difference between methylation status and expression profiles of genes because of two reasons 1) site to site variability of sample taken from placenta 2) rapid degradation of mRNA post-delivery in human placenta (Avila et al., 2010).

Nevertheless, tumour suppressor genes presumably play a significant role in placental development. Aberrant methylation pattern may be responsible for placental pathologies such as preeclampsia, intrauterine growth retardation, placental abruption and ischemia. Thus further work is warranted to compare the methylation status of these genes in the placenta of normal and abnormal pregnancies. In addition, the detection of differential methylation profiles in the DNA extracted from plasma samples of patients with abnormal pregnancies can be used as a predictive tool to detect abnormality early in pregnancy.

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