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Research Article

EVALUATION OF DNA METHYLATION OF FGFR2 GENE IN BREAST CANCER

Mina Dehbid[1*] and Hamidreza Vaziri[2]

[1]The National Institute of Genetic Engineering and Biotechnology, Pajoohesh Blvd., Tehran, Iran
[2]The University of Guilan, Rash, Guilan, Iran

ABSTRACT: DNA methylation is one of the Epigenetic marks at the cytosine residue of CpG dinucleotide. Epigenetic biomarkers can be used at any stage of a disease such as cancer, as diagnostic biomarkers, prognostic biomarkers and predictive biomarkers. Several genes have been identified as an Epigenetic biomarker in breast cancer. This study has evaluated breast cancer samples and normal samples to identify epigenetics biomarkers for breast cancer. DNA was extracted from peripheral blood of the 30 breast cancer samples and the 30 normal samples. Here was used a method for DNA methylation analysis that is based on digestion DNA with a methylation sensitive restriction enzyme and PCR amplification with gene-specific primers (MSRE-PCR method). FGFR2 gene is no significantly hypomethylated in breast cancer samples compared to normal samples which is analyzed with P value (P>0.05). Results of this study haven't showed any change in methylation of FGFR2 gene in breast cancer; nevertheless, more quantitative and detailed studies should be done in the future.

Keywords: Breast cancer; Epigenetic biomarker; MSRE-PCR method; FGFR2 gene

[*]Corresponding author: Mina Dehbid, The National Institute of Genetic Engineering and Biotechnology, Pajoohesh Blvd., Tehran, Iran, Tel: (+98) 21 44787301-27; E-mail: Armina_amd@yahoo.com

INTRODUCTION

Although, many of the researches are being allocated to clinical usage and detection of genetic, RNA expression markers and proteomic, DNA methylation is rapidly emerging as a new level of cellular information (Methylation Marker Discovery, 2015). DNA methylation is occurred in both gene promoter and non-promoter regions (Yegnasubramanian S, et al., 2011) by DNA methyltransferase enzyme. After DNA synthesis, enzyme transfers methyl from S-adenosylmethionine to cytosine (Laird PW, 2003).

In humans, the accurate expression of many tissue-specific, germline- specific, and X-chromosome-inactivation depends on DNA methylation (Manel Esteller, 2011). Many studies have shown that DNA methylation involves in regulation of gene activity, and also its disturbance associated with the diseases (Laird PW, 2003; Adorjan P, et al., 2002). Abnormal methylation of CPG leads to inactivation or overexpression of a wide variety of genes (Xue J, et al., 2013). Aberrant DNA methylation patterns such as hypermethylation and hypomethylation are associated with many types of cancer (Zhang FF, et al., 2011). Moreover, abnormal methylation is happened in CpG-rich regulatory elements in intronic and coding parts of genes for specific tumors. In recent studies have been reported that the hypermethylation of tumor suppressor genes and the hypomethylation of oncogenes. In addition, a correlation between increased gene expression and hypomethylation has been indicated for many oncogenes (Adorjan P, et al., 2002).

DNA hypomethylation was the primitive methylation abnormality knew in human cancer. DNA dimethylation during carcinogenesis may involve hemi methylated pairs as intermediates, led to extension of the loss of methylation on both strands (Ehrlich M, et al., 2009). Breast cancer is the most commonly recognized cancer among females (Ferlay J, et al., 2013). While many genetic mutations associated with breast cancer, scientific evidences suggest that methylation changes occur at the beginning and development of the disease (Sadikovic B, et al., 2008; Esteller M, 2008; Pogribny I, 2010), as well as changes of DNA methylation can be used in researches and clinics as biomarkers for diagnosis and prognosis in cancer (Herceg Z, et al., 2007). Different methods have confirmed methylation changes as hallmark of breast cancer (Hon GC, et al., 2012).

In recent years, the relationship between FGFR2 genes and breast cancer has been reported in several studies which have shown that FGFR2 involved in susceptibility to breast cancer [Bai A, et al., 2010; Hunter DJ, et al., 2007; Easton DF, et al., 2007). FGFR2 gene in human is also called K-SAM, CD332, BFR-1, TK25, TK1, KGFR, ECT1, CFD1, CEK3, BBDS, JWS،BEK which is located on the negative strand DNA and in cytogenetic region 10q26 (Zhang Y, et al., 1999).

The FGFR2 gene codes a protein called fibroblast growth factor receptor 2. This protein is one of four fibroblast growth factor receptors, which are related proteins that are involved in important processes such as cell division, regulation of cell growth and maturation, formation of blood vessels, wound healing, and embryonic development (Genetics Home References, 2013). FGF binding to FGFR leads to receptor dimerization, followed by receptor auto phosphorylation, which then activate downstream signaling pathways (Qing K, et al., 1999). Among these pathways, MAPK pathway involves in differentiation, angiogenesis (Ross MJ, et al., 2001), proliferation (LaVallee TM, et al., 1998) and migration of cells (Huang C, et al., 2004), and also AKT pathway involves in migration (Czaplinska D, et al., 2014) and survival of cells (Mavila N, et al., 2012) (Figure 1).

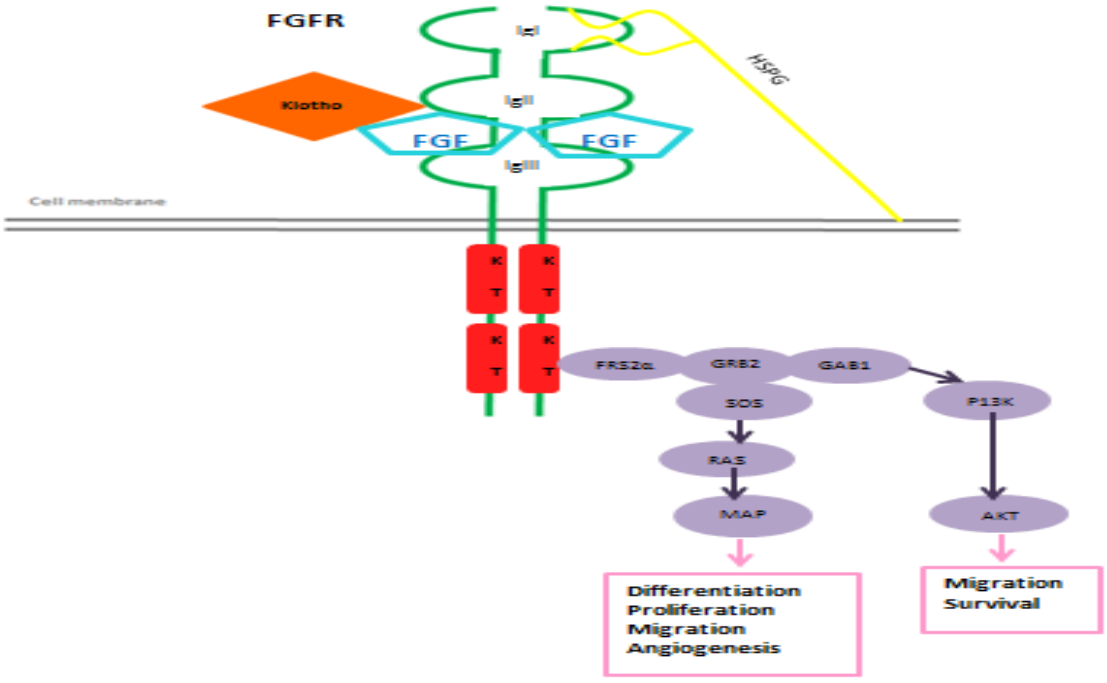

Figure 1: FGFR structure and downstream signaling. The extracellular domain of FGFR consists of three ligand-binding Ig domains. Binding of the ligand is stabilized by HSPG and Klotho. Intracellular tyrosine kinase domains (TK) are present. Upon ligand binding, the receptors dimerism, resulting in cross-phosphorylation of the tyrosine kinase domains. This leads to binding of several docking proteins, which can also be phosphorylated. Downstream signaling pathways: AKT, and MAPK.

MATERIALS AND METHODS

This study was approved by Ethics Committees of Imam Khomeini Hospital (for Blood sampling from breast cancer) and the National Institute of Genetic Engineering and Biotechnology (to do the research projects).

Sample collection and DNA isolation

Whole blood samples (Tubes containing EDTA (0.05M)) were collected from thirty normal women (As control samples) Breast cancer samples in 2013 at Imam Khomeini Hospital served as the study population, and stored at -70°C. Clinical data of the breast cancer samples are indicated in Table 1. Genomic DNA from 300 µl of whole blood of each sample was extracted using the GPP™ Solution. Measure of DNA purity and concentration are determined using a Nano Drop™ spectrophotometer at 280/ 260 nm.

Table 1: Clinic Data of 30 breast cancer patients.

Clinic pathological features		Breast cancer patients % (n)
Age range of patients		28-80
Stage of Breast cancer	I II III	13.3% (4) 53.3% (16) 33.3% (10)
ER[1]	positive negative	63.3% (19) 36.6% (11)
PR[2]	positive negative	60% (18) 40% (12)
HER2[3]	positive negative	50% (15) 50% (15)

[1]Estrogen receptor, [2]Progesterone receptor, [3]Human epidermal growth factor receptor-2.

Methylation Sensitive Restriction Enzymes (MSRE-PCR)

This method makes it possible to detect allelic-specific methylation of FGFR2 gene (Melnikov AA, et al., 2005). Digestions were performed with Nae I (recognition site 5'GCCGGC3'; Jena Bioscience). It cuts the CAC↓GTG sequence, although cleavage site is blocked by methylation. Therefore, the enzyme does not cut the methylated form of CA5mCGTG sequence. Typically, 300 ng of genomic DNA were mixed with 1 µl of 10XL Buffer and digested with 0.3 µl of the enzyme at 37°C for overnight (To ensure complete digestion) and By the sterilized distilled water volume of the reaction was raised to 10 µl.

Normal samples and breast cancer samples were treated by Nae I enzyme in the same method. Primer design was done using primer3 software for genomic fragment containing recognition site of enzyme. For breast cancer and normal samples, PCR amplification was served on untreated and treated DNA with the Nae I enzyme. PCR was performed with these conditions: The 20 µl reaction mixture, consisting of DNA (225 ng), 0.5 µl of forward and reverse primer (5 pmol/µl), 1 µl of DMSO and 10 µl of Taq 2x master mix were used in PCR. 1 cycles of 95°C for 5 minutes followed by 35 cycles of 95°C for 45 seconds, 60.9°C for 30 seconds, 72°C for 30 seconds, and 1 cycles of 72°C for 7 minutes.

Statistical Analysis

In order to statistical analysis of differences in methylation's study region of FGFR2 gene between 30 breast cancer samples and 30 normal samples by The SPSS version 21.0 software(SPSS, Inc., Chicago, IL) was used chi- square test analyze. P-Value <0.05 was considered to be Significant.

RESULTS

300 ng of extracted DNA from breast cancer samples and normal samples were digested with Nae1 enzyme. Following, undigested DNA and digested DNA with Nae1 enzyme were used as a template of PCR (Figure 2).

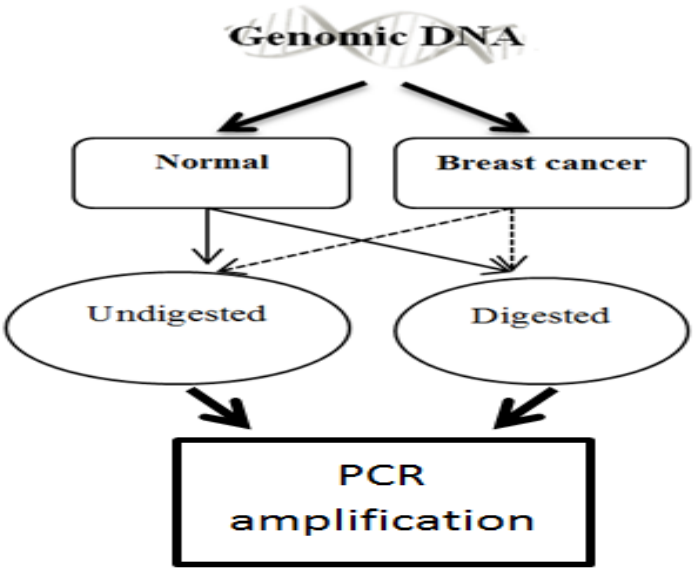

Figure 2: Pattern of experiment by MSRE-PCR.

For the 4 types of template of PCR (digested and undigested breast cancer samples, and digested and undigested normal samples), separate PCR reactions were performed using primers flanking GCCGGC sites in FGFR2. In order PCR products of FGFR2 gene were electrophoresed on a 1.8% agarose gel, stained with ethidium bromide, and photographed using Gel-doc system. A typical example is indicated in Figure 3 as a quality control for PCR reactions (35 cycles) that demonstrated accuracy of amplification of considered fragment.

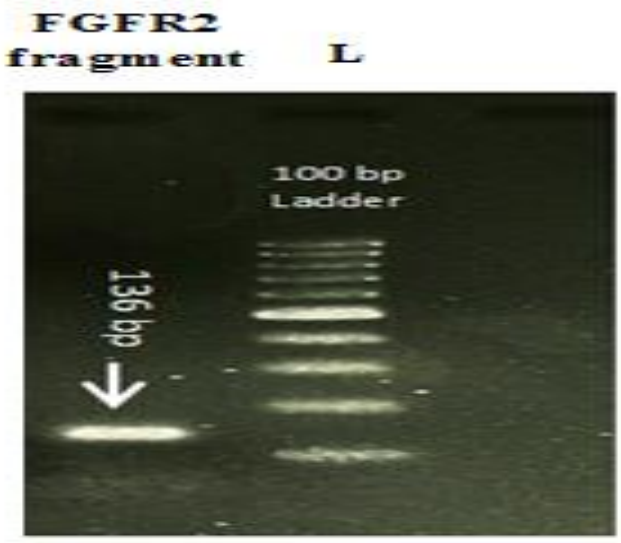

Figure 3: Quality control for PCR reactions. The PCR reaction products of FGFR2 gene are certified by electrophoresed on 1.8% agarose gel (Lane right to left: ladder 100 bp and 136 bp FGFR2, respectively).

The Presence of PCR products in breast cancer and normal samples of treated by enzyme can show methylation in FGFR2 gene and its insensitivity to the enzyme. 1#, 2# and 3# breast cancer samples, and 1# and 2# normal samples evaluated by MSRE-PCR as shown in Figure 4.

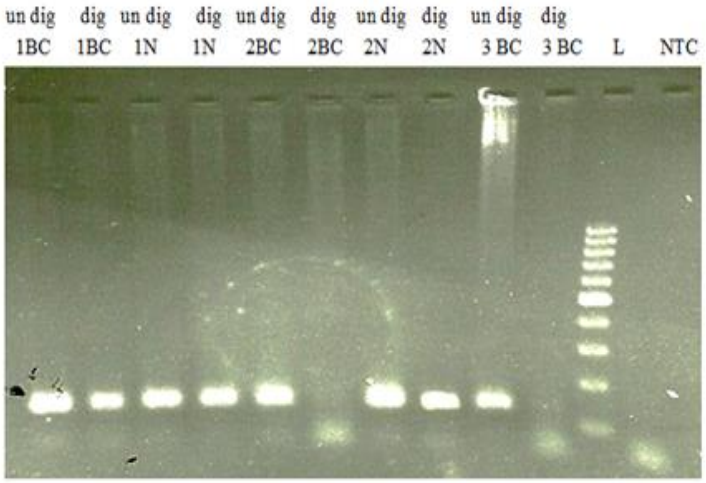

Figure 4: Agarose gel (1.8%) electrophoresis of PCR products of FGFR2 gene in breast cancer and normal samples. The absent of band in sample#2 and sample #3 of digested breast cancer indicates un methylation of FGFR2 gene, but the present of band in sample#1 of digested breast cancer, and 1# and 2# normal samples indicates methylation of FGFR2 gene. (UN dig: undigested, dig: digested, BC: breast cancer, N: normal, L: ladder, NTC: no template control).

The absent of band in indicates unmethylation of FGFR2 gene, but the present of band in samples indicates methylation of FGFR2 gene. Bands in the digested sample were compared with bands in the matching undigested sample for both groups of normal and breast cancer (Table 2). The results indicate that there is no statistically significant difference between the control group and the patient group.

Table 2: Results of MSRE-PCR in status of template digested.

PCR product in digested samples	Normal Sample % (n)	Breast cancer Sample % (n)
absent of PCR product	43.3% (13)	63.3% (19)
present of PCR product	56.7% (17)	36.7% (11)
Total samples	100% (30)	100% (30)

DISCUSSION

DNA hypomethylation was the primitive epigenetic abnormality identified in human tumors. The association of global or focal DNA hypomethylation with the early stages of carcinogenesis or with tumor progression provides cancer markers that should be very useful in the clinic (Ehrlich M, 2009). Specially, Epigenetic markers based on abnormal DNA methylation, can be used to diagnose cancer (Grunau C, et al., 2008). Bodily fluid including peripheral blood can be used as biomarker for DNA methylation analysis (Mikeska T, et al., 2014). Blood-based specimens such as cell-free circulating nucleic acid and DNA extracted from leukocytes in peripheral blood may be a potential source of noninvasive cancer biomarkers (Lian Li, et al., 2012).

In this study, we extracted DNA of peripheral blood cells in order to discover the epigenetic diagnosis biomarker in breast cancer. We used a method for DNA methylation analysis in FGFR2 gene that is based on digestion DNA with a methylation sensitive restriction enzyme and PCR amplification of Surrounded fragment. If the fragment under study is unmethylated, no PCR product is observed after digestion. However, if the fragment is methylated product can be seen.

MSRE-PCR Detects cytosine methylation at a single nucleotide site and it is similar to almost every method of methylation analysis (for example: COBRA (Mikeska T, et al., 2014) and Methyl Quant (Thomassin H, et al., 2004) because this method has two steps.

We used Nae I (recognition site 5'GCCGGC3'), with good survival in the reaction at 37°C for overnight and enough activity on tested samples (digestion of 300 ng of genomic DNA in 10 µl reaction). After treatment step by Nae I (methylation sensitive restriction enzyme) in MSRE method, amplification of treatment and untreatment templates can be done in breast cancer and normal samples using gene-specific primers. Primers were de amplified fragments of 136 bp (If the fragment is methylated).

In this study, we have observed two types of results using MSRE method: presence or absence of a band in the breast cancer and normal samples with treated template by methylation sensitive restriction enzyme in MSRE-PCR method (Table 2). According to previous studies, including:

In 2013 by Heyn et al. has demonstrated that there are differences in methylation between breast cancer and normal samples in several genes such as FGFR2 gene. Their research indicated hypomethylation of the FGFR2 gene in breast cancer (Heyn H, et al., 2013). In 2010 by Turner, et al., has showed that signaling FGFR2 because its overexpression can prevent apoptosis, which is a characteristic of cancer, cells (Turner N, et al., 2010). 34. Stepanova, E., et al. (2013) to prove the key role of FGFR in the process of tumor angiogenesis studies done on this issue. Their results showed that the inhibition of FGFR2 effects on endothelial cell proliferation and formation of mature vessel (Stepanova E, et al., 2013).

According to studies, the authors concluded that hypomethylation of promoter region as an important regulatory region can lead to activation of FGFR2 gene and high expression of it in breast cancer. High expression of the gene can lead to angiogenesis and inhibit of apoptosis, which is characteristic of cancer cells.

Although many studies have identified the role of genes in breast cancer, FGFR2 gene is probably a good candidate for study on breast cancer. Unfortunately, in our study indicated FGFR2 gene is no significantly hypomethylated in breast cancer ($P>0.05$). We could not introduce FGFR2 gene as a diagnostic biomarker for breast cancer. Therefore, the use of more precise quantitative methods can be more efficient.

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REFERENCES

Adorján P, Distler J, Lipscher E, Model F, Müller J, (2002). Tumour class prediction and discovery by microarray-based DNA methylation analysis. Nucleic Acids Res 30: e21.

Bai A, Meetze K, Vo NY, Kollipara S, Mazsa EK, (2010). GP369, an FGFR2-IIIb–Specific Antibody, Exhibits Potent Antitumor Activity against Human Cancers Driven by Activated FGFR2 Signaling. Cancer research 70: 7630-7639.

Czaplinska D, Turczyk L, Grudowska A, Mieszkowska M, Lipinska AD, (2014). Phosphorylation of RSK2 at Tyr529 by FGFR2-p38 enhances human mammary epithelial cells migration. Biochim Biophys Acta 1843: 2461-2470.

Easton DF, Pooley KA, Dunning AM, Pharoah PD, Thompson D, (2007). Genome-wide association study identifies novel breast cancer susceptibility loci. Nature 447: 1087-1093.

Ehrlich M (2009). DNA hypomethylation in cancer cells. Epigenomics 1: 239-259.

Epigenomics AG (2014). Methylation Marker Discovery. Matthias Schuster. Berlin.

Esteller M (2011). Epigenetic changes in cancer. F1000 Biol Rep 3: 9.

Esteller M (2008). Epigenetics in cancer. N Engl J Med 358: 1148-1159.

Ehrlich M (2009). DNA hypomethylation in cancer cells. Epigenomics 1: 239-259.

Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, (2015). Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 136: E359-386.

Genetics Home References (2013). FGFR2. Guide to genetics conditions.

Grunau C, Brun ME, Rivals I, Selves J, Hindermann W, (2008). BAGE hypomethylation, a new epigenetic biomarker for colon cancer detection. Cancer Epidemiol Biomarkers Prev 17: 1374-1379.

Heyn H, Carmona FJ, Gomez A, Ferreira HJ, Bell JT, (2013). DNA methylation profiling in breast cancer discordant identical twins identifies DOK7 as novel epigenetic biomarker. Carcinogenesis 34: 102-108.

Herceg Z, Hainaut P (2007). Genetic and epigenetic alterations as biomarkers for cancer detection, diagnosis and prognosis. Mol Oncol 1: 26-41.

Hon GC, Hawkins RD, Caballero OL, Lo C, Lister R, (2012). Global DNA hypomethylation coupled to repressive chromatin domain formation and gene silencing in breast cancer. Genome Res 22: 246-258.

Hunter DJ, Kraft P, Jacobs KB, Cox DG, Yeager M, et al., (2007). A genome-wide association study identifies alleles in FGFR2 associated with risk of sporadic postmenopausal breast cancer. Nature genetics 39: 870-874.

Huang C, Jacobson K, Schaller MD (2004). MAP kinases and cell migration. J Cell Sci 117: 4619-4628.

Laird PW (2003). The power and the promise of DNA methylation markers. Nat Rev Cancer 3: 253-266.

LaVallee TM, Prudovsky IA, McMahon GA, Hu X, Maciag T (1998). Activation of the MAP kinase pathway by FGF-1 correlates with cell proliferation induction while activation of the Src pathway correlates with migration. J Cell Biol 141: 1647-1658.

Li L, Choi JY, Lee KM, Sung H, Park SK, (2012). DNA methylation in peripheral blood: a potential biomarker for cancer molecular epidemiology. J Epidemiol 22: 384-394.

Mavila N, James D, Utley S, Cu N, Coblens O, (2012). Fibroblast growth factor receptor-mediated activation of AKT-Î²-catenin-CBP pathway regulates survival and proliferation of murine hepatoblasts and hepatic tumor initiating stem cells. PLoS One 7: e50401.

Melnikov AA, Gartenhaus RB, Levenson AS, Motchoulskaia NA, Levenson Chernokhvostov VV (2005). MSRE-PCR for analysis of gene-specific DNA methylation. Nucleic Acids Res 33: e93.

Mikeska T, Craig JM (2014). DNA methylation biomarkers: cancer and beyond. Genes 5: 821-864.

Pogribny IP (2010). Epigenetic events in tumorigenesis: putting the pieces together. Exp Oncol 32: 132-136.

Qing K, Mah C, Hansen J, Zhou S, Dwarki V, (1999). Human fibroblast growth factor receptor 1 is a co-receptor for infection by adeno-associated virus 2. Nat Med 5: 71-77.

Ross MJ, Claesson-Welsh L (2001). FGF and VEGF function in angiogenesis: signalling pathways, biological responses and therapeutic inhibition. Trends in pharmacological sciences 22: 201-207.

Sadikovic B, Al-Romaih K, Squire JA, Zielenska M (2008). Cause and consequences of genetic and epigenetic alterations in human cancer. Curr Genomics 9: 394-408.

Stepanova E, Solomko E, Ryabaya O, Peretolchina N, Tsimafeyeu I, (2013). Targeting angiogenesis driven by fibroblast growth factor using RPT835, an FGFR2 inhibitor. Journal of Clinical Oncology. AMER SOC CLINICAL.

Thomassin H, Kress C, Grange T (2004). MethylQuant: a sensitive method for quantifying methylation of specific cytosines within the genome. Nucleic Acids Res 32: e168.

Turner N, Lambros MB, Horlings HM, Pearson A, Sharpe R, (2010). Integrative molecular profiling of triple negative breast cancers identifies amplicon drivers and potential therapeutic targets. Oncogene 29: 2013-2023.

Xiong Z, Laird PW (1997). COBRA: a sensitive and quantitative DNA methylation assay. Nucleic Acids Res 25: 2532-2534.

Xue J, Wijeratne SS, Zempleni J (2013). Holocarboxylase synthetase synergizes with methyl CpG binding protein 2 and DNA methyltransferase 1 in the transcriptional repression of long-terminal repeats. Epigenetics 8: 504-511.

Yegnasubramanian S, Wu Z, Haffner MC, Esopi D, Aryee MJ, (2011). Chromosome-wide mapping of DNA methylation patterns in normal and malignant prostate cells reveals pervasive methylation of gene-associated and conserved intergenic sequences. BMC Genomics 12: 313.

Zhang FF, Cardarelli R, Carroll J, Zhang S, Fulda KG, (2011). Physical activity and global genomic DNA methylation in a cancer-free population. EPIGENETICS 6: 293-299.

Zhang Y, Gorry MC, Post JC, Ehrlich GD (1999). Genomic organization of the human fibroblast growth factor receptor 2 (FGFR2) gene and comparative analysis of the human FGFR gene family. Gene 230: 69-79.

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