

Breast Cancer and Smell: Hints from Epigenetic and Functional Alteration of the Olfaction

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Abstract

Purpose: Olfactory receptors are G protein coupled surface receptors (GPCRs) of which their ectopic expression is currently of mounting interest to the development and metastasis of malignancies. These genes having a direct contact with the environment may probably be stimulated by various factors which can bring about methylation aberrations, including DNA hypo and hyper-methylation. Here we gather clues from epigenetic and phenotypic data in order to further our understanding of the potential association of the olfaction with oncogenesis.

Methods: Whole methylome dataset of breast cancer series generated by Illumina Infinium Human Methylation 450 Bead Chip was interrogated for differentially methylated genes and further subject to network analysis using various search tools. Analysis of putative phenotypic trait in olfaction function was performed using smell detection and smell identification tests and data was analyzed using Mann-Whitney test.

Results: Sixty-eight differentially methylated ORs were enriched mainly on chromosomes 1q23, and 11p15, specifically 1q44 (P value $6.867e-20$). Amongst the disease signatures of these hypomethylation events was breast cancer itself (P value 0.004437). Network analysis suggests the interaction of differentially hypo and hyper methylated olfactory receptor genes might be pivotal in stimulating several important biological pathways including circadian genes and pathways potentially associated with metastasis. Phenotypic smell test shows a generalized impairment of smell capability in breast cancer patients as compared to controls (Mann-Whitney Test $P=0.0001$), an effect that is independent of chemotherapy

Conclusions: The olfaction appears as a crucial element in carcinogenesis, evident by both phenotypic and genotypic (epigenetic) data in a well characterized breast cancer subset.

Background

Breast cancer, a multi-factorial and multiple phenotypic disease, is the leading cause of cancer death among females worldwide [1]. In sub-Saharan countries, young women are suffering from a rapidly accelerating burden of aggressive breast cancer of unknown etiology with few studies on its biology [2]. In the heart of the complex causation and etiology of cancer is the interaction of genetic predisposition and the environment. DNA methylation leading to epigenetic modification is thought to play an important role in environmentally driven alterations known to be associated with chronic human diseases such as cancer, autoimmune and neurodegenerative illnesses [3, 4]. Of the several cellular molecules that are in direct contact with the environment and might be more affected and aberrantly methylated by adverse environmental factors are the olfactory receptors (ORs). ORs are G-protein-coupled surface receptors (GPCRs) and are encoded by the largest multi-gene family in the human genome comprising greater than 800 genes of which about half are believed to be functionally engaged in signal transmission [5, 6] Studies revealed high level of single nucleotide polymorphisms in promoter regions of ORs to cause

variability of olfactory cognition of numerous perceived environmental stimuli [7, 8], consistent with these receptors being under evolutionary pressure, and given their function and involvement in various aspects of human biology including non-olfaction functions [9-12]. Flegel and his group revealed the expression of about 28% out of the functional ORs within the human genome in ectopic tissue, and OR51E1, OR51E2, OR2A1 were commonly expressed in various tissues in their samples [13]. Functionally, ORs are known to guide sperm chemotaxis [14], to regulate migration and adhesion of muscle cells [15] to have a role in cell positioning during embryogenesis and cell-cell recognition [16], thus influencing reproduction and other vital physiological functions [17]. The association of ORs with different malignancies and its involvement in promotion of cell invasion and metastasis has been reported [18]. Over expression and enhanced invasiveness of OR-expressing cancer cells has been reported as well following in-vitro stimulation by odorants [19]. In a study by Muranen et al., [20], over expression of OR genes have been shown in breast tumors of which almost half the expressed ORs resided on chromosome 11. A recent mouse study has shown that intra-ovarian follicular olfactory receptors and not the olfactory neurons respond to ligands present in the environment and manipulate the reproductive functions [17].

Given the above, and previous reported enrichment of OR in various cancer genetic and epigenetic data sets including ours [21, 22], we aim to study such alterations in one of the few available sets of breast cancer epigenomes and continue exploring possible links between genome wide methylation and breast cancer [23] with emphasis on OR genes and their potential interaction with other breast cancer genes.

Materials And Methods

Phenotypic Study

Olfaction function test was performed in a hospital setting on 32 breast cancer cases and 38 healthy controls to assess the smell impairment associated with breast cancer. Patients with any disease known to affect olfactory dysfunction such as problem in the nasal cavity and neurological disorder were excluded. Participants were considered as normal if they reported no olfactory dysfunction. Briefly, assessment of olfaction in breast cancer patients and controls used questionnaire to interview all subjects followed by olfaction assessment using smell detection and smell identification tests. In smell detection the odorants were selected to suit Sudanese local dietary and cultural habits. Smell identification test was performed by modifying the method by Gupta [24]. In the modified method 16 local odorants were selected for multiple forced choices from four verbal items per test odorant. In smell detection test we used the standard ' Alcohol Sniff Test (AST) [25]. Standard 70% alcohol pad was used. The pad was moved upward 1 cm per exhalation until the participant reported detection. The further distance of the odorant from the nose that the subject can detect was defined as the threshold. Since this test is commented upon due to alcohol's trigeminal effect, we also performed a coffee sniff test (CST) as well. For Statistical analysis all relevant data from the phenotypic study: the demographic data and other required information from the questionnaire, and the results of the smell tests were collected, tabulated and statistically analyzed. Mann-WhitneyTest, a nonparametric test for the significance of the difference

between the distributions of two independent samples was used to test differences in olfactory score between the cases and controls and the results were plotted using the program Plotly.

We analyze a data set of 16 DNA whole methylome, eight of primary breast tumors and eight matched controls from adjacent healthy tissues using the Illumina Infinium Human Methylation 450 (HM450) [Infinium] Bead Chip array from the Beijing Genomics Institute (BGI). This is mainly to corroborate earlier findings on the role of epigenetic silencing from a larger well characterized data set of breast cancer from Sudan [21]. The Genome-wide DNA differential methylation analysis findings are published online in bioRxivDoi: 10.1101/03432 and Abdalla et al., [23].

The functional gene-annotation enrichment and pathway analyses of the significantly enriched genes within the differentially hypomethylated genes was obtained using a network analysis data base (STRING), as well as Enrichr, a functional annotation tool. This database performs functional analysis of large gene lists using information from GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes), Biocarta, Reactome databases. The GIFtS value for a subset of all 31 genes which are completely hypomethylated at the promoter site was calculated. A GIFtS value for a gene is defined as the number of GeneCards sources, out of a total of 68 sources that include information about this gene [26].

Results

In the pilot experiment to analyze putative phenotypic trait for olfactory function in breast cancer, results indicate a clear difference (Mann-Whitney Test $P=0.0001$) in the smell sensitivity between cases and controls (Figure 1). The general trend was an inferior smell capacity in both treated and untreated cases as compared to controls. Patients under chemotherapy treatment presented with intermediate values between the cases and controls. A qualitative test concurs with a smell impairment with patients showing impaired odorant identification ability compared to controls in all of the test odorants. The most significant observation seen was with 'Mjmoh' with respect to the other odorants (ginger, lemon and daqua) P -value 0.001, 0.001 and 0.002 respectively by Spearman's rho correlation (Table 1).

Whole methylome analysis of the 16 breast tissues samples revealed a total of 2,811 genes as differentially hypomethylated in cancer samples as compared to the adjacent non-cancerous tissue; of those 69 Olfactory (OR) genes were found, rendering the olfactory receptors among the most prominently differentially hypomethylated loci at different sites and 13 olfactory genes were differentially hypermethylated (Table 2 and Table 3). A majority of the completely hypomethylated ORs were aggregated on 1st exon site (Figure 2). Significant enrichment of differentially hypomethylated OR family 2 genes (19 genes) was shown to aggregate within chromosomes 1 and 11 (Figure 2), specifically mapped to 1q44 (p -value $6.867e-20$). That was also the case for hypermethylated ORs (P value $1E-05$). A snapshot of a region in chromosome 1, depicts an example of the patterns and intensity of differential hypomethylation measured in Beta-Values (Figure 3). Two genes (OR2W5 and OR2L13) were both

hypermethylated and hypomethylated at chromosome1q44. Other highly significant locations shared by both hypomethylated and hypermethylated ORs were 11p15, 11q24, 11q11.

Further filtration was done focusing on the promoter site only and found a set of 10 olfactory genes completely hypomethylated at the aforementioned site; (members of G Protein-Coupled Receptors (GPCRs) of which 8 were ORs (OR2T10, OR10Z1, OR2T6, OR6Y1, OR52N2, OR52E4, OR4K5, OR14J1). Of the remaining two, one was vomero-nasal 1 receptor 4 (VN1R4) and the other was a G protein-coupled receptor 62 (GPR62), Table 4. Four of those were at chromosome 1q and two were at 11p (Table 2 and Figure 2). Moreover, besides the promoter, all except OR52E4 and OR52N2 were also hypomethylated at TSS1500 site. None of these 10 were hypomethylated at 3'UTR, 5' UTR and Body sites.

Pathway analysis of this set was carried out using Enrichr tool. The top three significant pathways were cell communication (KEGG), translation Factor (WIKI), interaction between L1 and Ankyrins (REACTOME) with P-values < 0.05. Pathway enrichment based on 31 genes out of 2811 genes (Table 1) including the 10 olfactory genes completely hypomethylated in the promoter site , revealed olfactory transduction (P-value of 0.004330) as one of the pathways, calcium signaling, cell communication and MAPK were also enriched but were not significant and genes enriched in this term were OR10J1, OR1G1 and OR2J3) (Figure 4), while pathways associated with cell invasion such as regulation of actin cytoskeleton, (P-value, 2.58 e-06), focal adhesion (P-value 7.87 e-08) signal transduction (P-value 5.95e-11) were significantly enriched with differentially hypermethylated genes .

Enquiry of all 68 hypomethylated OR gene set using Enrichr tool revealed disease signatures associated with breast cancer_GSE3744 (P-value 0.004437), OR10J1;OR1G1;OR2J3 (Table 2). PPI analysis of all hypomethylated OR genes using STRING tool, two sub-networks were found to directly interact with the core sub-network of hypomethylated ORs: one comprises phototransducer genes such as GNAT1, GNB5, RHO, PDE6A which were linked to the ORs through GNGT1 gene and the second sub network included interacting genes such as STIM, ORAI and ITPR linked to the ORs through GNB1 gene Similarly, network interaction of hypomethylated OR genes and selected genes which were highly enriched using STRING and Enrichr tools reveals important cancer genes.

When analysis was carried out using all differentially hypomethylated and hypermethylated OR genes as query genes a sub-network was revealed in which ORs were seen to interact with genes encoding subunits of guanine nucleotide-binding proteins, known to integrate signals between receptors and effector proteins; Adenylatecyclases (ADCY) and phosphodiesterases (PDE) regulate cyclic nucleotides and thus are involved in cell signaling and its mutation is known in various disorders such as the brain and endocrine (hormone) systems, RGS9BP and RHO were connected through GNAL, GNB1, GNGT1.

Discussion

The contribution of epigenetics to malignancy has been dominated by the paradigm of hyper-methylation of tumor-suppressers and hypomethylation of oncogenes, where the expression of oncogenes is associated with hypomethylation of their CpG sites and tumor suppressors being inactivated through

hypermethylation [27-29], although this has been challenged and hypomethylation is claimed to occur later than hypermethylation during cancer development and is an indicator of a more advanced and metastatic tumor [30]. Additionally, based on meta-analysis, hypomethylation was found to associate with worse survival of cancer patients. In ovarian cancer, it is associated with tumor advancement and is an indicator of poor prognosis [31,32].

Although the sense of smell is one of the most fundamental interfaces with the environment, the major role of ORs in detection of volatile odorant by olfactory sensory neurons [33], their ectopic expression in non-olfactory tissues and their association with human diseases including malignancies [18] is still not well understood.

The result of the phenotypic test although remarkably distinctive of cases and controls was not due to conventional gain of phenotypic function due to hypomethylation in epigenetic terms. Rather the outcome was an impaired sense of smell which is more consistent with the olfaction being part of the neuronal signaling modality, where hypermethylation fosters neuronal function. This trend does not seem to be masked by the interference of chemotherapy known to affect the sense of smell and taste. The hypomethylation seen here, thus, in addition of being a tissue specific mechanism involved in carcinogenesis might have an innate germ line (inherited) origin and characteristics that may be detected as a phenotype. It might be the fact that the OR genes are primarily G-protein-coupled receptors (GPCRs) that explains their wide functionality including their role in cancer. They are known to be involved in many physiological processes including response to environmental stimulants and are currently the target of many chemotherapeutic agents. Cancer cells are known to hijack body's physiological processes for their own survival benefit and thus their communication with GPCRs is inevitable. The incrimination of GPCRs in tumor progression and metastasis is well reported [18,34].

Most hypomethylation of OR genes were at 1st exon. First exon methylation is believed to block transcript initiation and hypomethylation of the first exonic region after treatment with decitabine is associated with transcriptional activation [35]. Testis specific, MHC-linked OR2H1 gene [13] was found in our breast cancer samples differentially hypomethylated at 5' UTR and promoter site. Six of the differentially hypomethylated ORs genes (OR7C2, OR7D4, OR10Z1, OR10G9, OR14C36, and OR1A2) were previously reported as genes with high SNP diversity at their promoters [7].

Though, ORs are found distributed across all 21 human chromosomes [36]; the highly significant hypomethylated OR genes were located on chromosomes 1q and 11p. Where 60% of the GPCR genes were housed and these chromosomal arms are known to be associated with cancer development [37]. Significant enrichment of the olfactory receptor family two members which were hypomethylated in the current samples were mapped to chromosomes 1q specifically to chr1q44 (P value, 6.867e-20) and this location is reported to be one of the viral integration sites [38]. Breast cancer is reported to have Epstein Barr virus as main viral etiology in Sudanese [21]. Two genes (OR10Z1 and OR6Y1) were located at 1q23.1, According to various studies chromosome 1 aberrations are associated with different cancers such as neuroblastoma [39], cervical [40] and colorectal [41]. In breast cancer, gains at 1q are found in

over 50% of breast tumors [42]. Based on frequent karyotypic changes in chromosome 1 in breast cancer Chen et al., [43] proposed that the development of breast cancer might be caused by inactivation of a gene (s) located on 1q23-32.

The expression of some ORs is reported to be controlled by odorants which are dually active; OR2A1 is known to be regulated by sandalwood compounds which initiate neuronal signaling as well as hormonal transcriptional control of specific genes. [45,44]. OR51E2/PSGR, a marker of prostate cancer and OR7D4 can be activated by androstenone [46-50], also, OR51E1 is reported to associate with gastrointestinal neuroendocrine carcinomas [51], It can be activated by steroid hormones [13]. All three OR7D4,OR51E2/PSGR and OR51E1 were hypomethylated in our samples.

Busse and his group demonstrate that the 'cutaneous' OR, (OR2AT4) within the keratinocytes has a major role in response to environmental stimuli and when stimulated by its agonist sandalore, a synthetic sandalwood odorant, it promotes proliferation and migration of keratinocytes and thus it has positive impact on wound healing [52]. It may be plausible to assume therefore, that breast ORs may directly communicate with the environment such as volatile organic compounds which may stimulate the hormones and eventually contribute to carcinogenesis. In a recent study, the ovary of a BRCA1 mutated mice was found to mediate smell signals without the help of the nose and influence estrous cycle [17]. Interestingly Insulin was found to alter the olfactory nerve evoked excitatory postsynaptic currents in mitral cells, thus providing a link between the hormonal, nutritional systems and the olfaction [53].

In conclusion and based on the above, hypomethylated olfactory receptor genes may be associated with breast cancer and support the hypothesis that ectopic olfactory genes may have a role in tumorigenesis. Some of the identified set of olfactory genes warrants further investigation as experimental evidence is required to reveal the influence of 'human Odorome' in breast cancer in particular, the impact of methylated olfactory receptor genes on hormone function as well as its possible cross talk with the phototransducer genes.

Declarations

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Availability of data and material: The data will be available in Sudan National Genome project upon request or from corresponding author directly.

Authors' contributions:GF, HSM and MEI conceived of the study and wrote the manuscript. GF performed laboratory experiments and bioinformatics analysis together with MOE and RSK. KHM recruited the patients, AK performed the smell test.

Ethics approval: Ethical approval for this study was granted by the ethical committee, Institute of Endemic Diseases (IEND), University of Khartoum (U of K), Sudan.2018.

Consent to participateSigned informed consents were obtained from each subject in the study.

Consent for publication Written consent to publish this information was obtained from parents and it is available upon request.

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Tables

Table 1: OR families within the top six highly significant chromosome locations with associated genes of all hypomethylated OR genes

Chromosome	P-value	Gene Within	Remark
chr1q44	.867e-20	OR2G6;OR2M5;OR2M3;OR2M2;OR2T6; OR2T33;OR2T11;OR2T3;OR2T2;OR2W5; OR2L13;OR2M1P;OR2B11	All 13 genes are within olfactory receptor family 2
chr11p15	1.055e-10	OR51F2;OR51E1;OR52J3;OR56A5;OR52E4; OR51S1;OR51B5;OR5P2;OR10A4;OR51I1; OR51A7;OR52N2	Nine out of 12 are within olfactory receptor families 51 and 52
chr1q23 family 10	5.089e-10	OR10J1;OR10Z1;OR10J3;OR10X1;OR10J5; OR6K3;OR10T2;OR6P1	Six out of 8 are within olfactory receptor
chr11q11	0.000002564	OR5W2; OR8K1; OR5L1; OR4C16;OR4A15	Four out of 5 are within families 4 and 5
chr17p13 family 1	0.0004215	OR1A2;OR1A1;OR1E2;OR1G1;OR3A2	Four out of 5 are within olfactory receptor
chr11q24 family 10	0.001939	OR10G4;OR10S1;OR10G9	All three genes are within olfactory receptor

Table2: Top four disease signature of completely differentially hypomethylated OR genes and associated genes with P-value based on enquiry of 68 hypomethylated OR gene set using Enrichrtool.

Disease	P-value	Genes
Psoriasis vulgaris	1.66E-05	OR10J1; OR14J1; OR5P2; OR7C2; OR51I1
Idiopathic fibrosingalveolitis	0.004437	OR1A1; OR5V1; OR14J1; OR12D2; OR51I1
Breast Cancer	0.004437	OR10J1; OR1G1; OR2J3
Huntington's Disease	0.044635	OR5V1; OR10A4

Figures

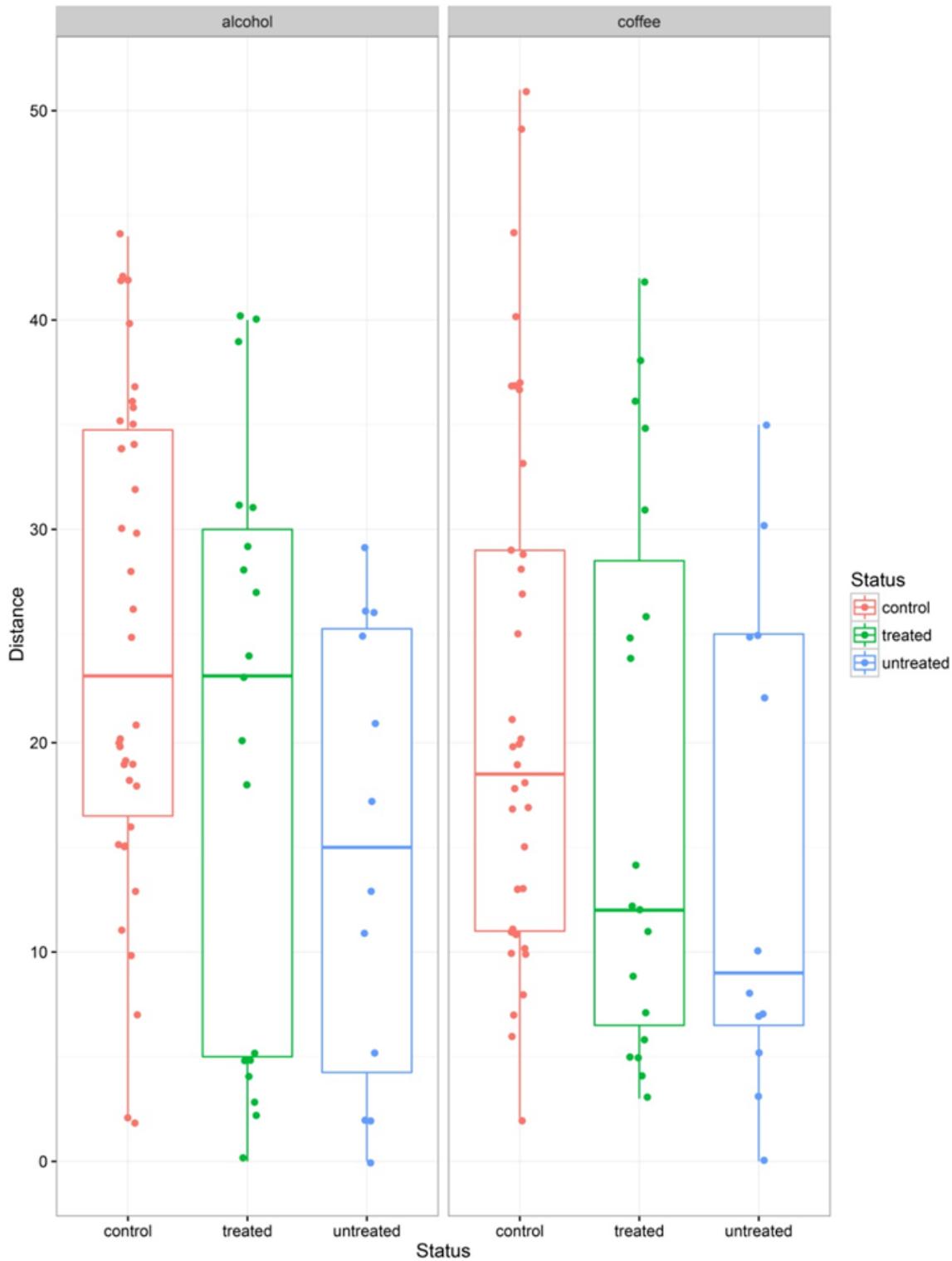
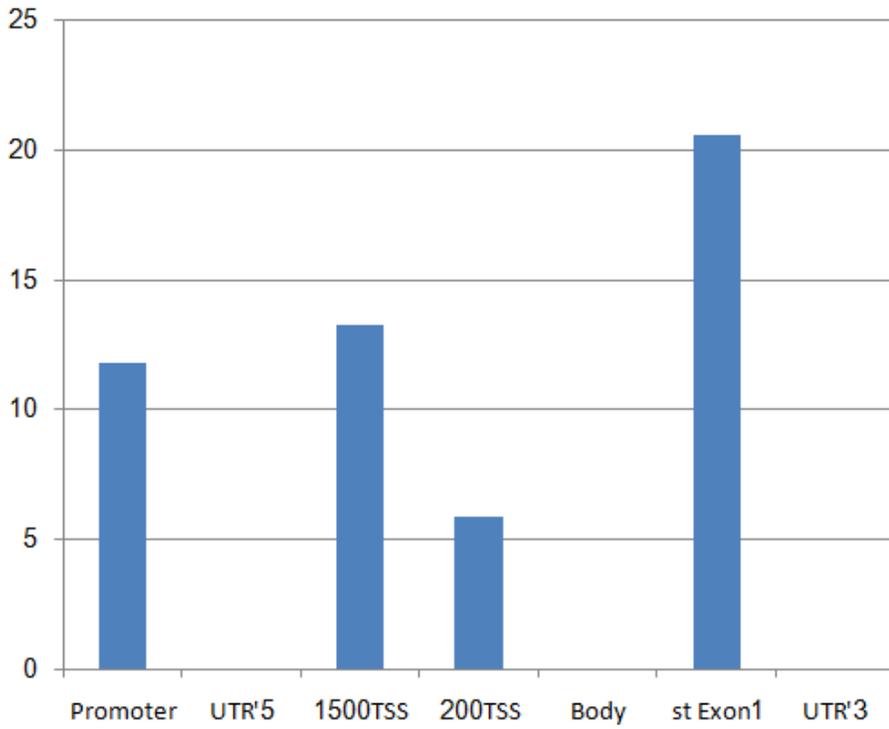
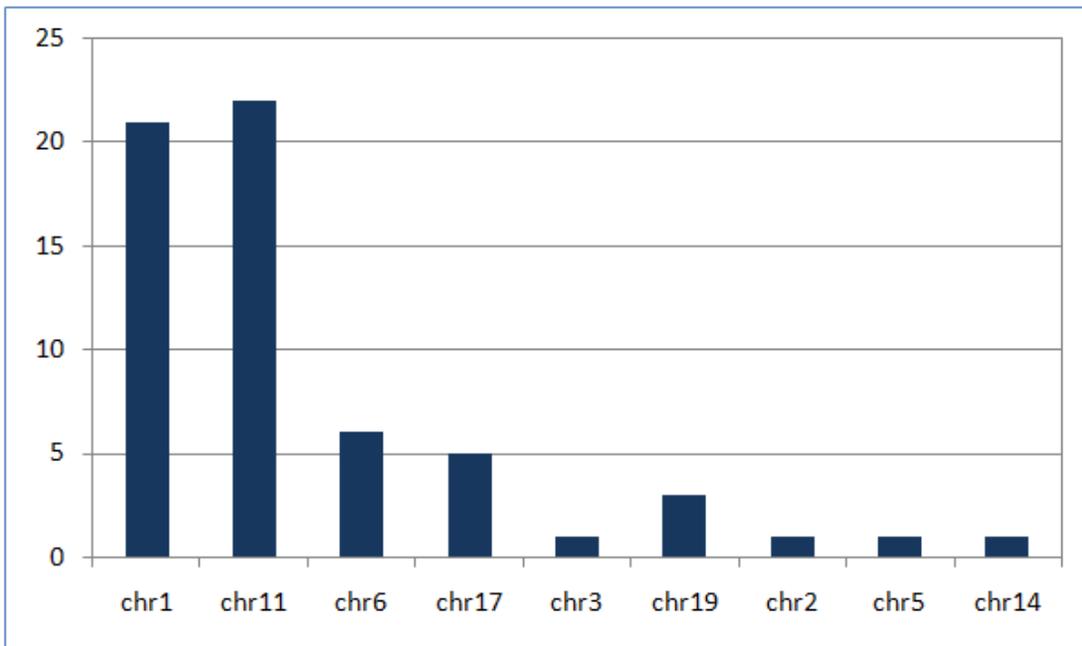


Figure 1

Distributions of olfactory scores (smell) in two independent samples of breast cancer (blue) and healthy controls (red). Mann-Whitney Test a nonparametric test was used to calculate the significance of the difference between the two groups. Y axis distance in cm from origin of test, X individuals.



a



b

Figure 2

a: Distribution of completely differentially hypomethylated olfactory receptor genes, histogram indicates 1st exon to house most of these genes. b. Histogram of the chromosomal location of the hypomethylated OR genes. Most are within chromosome 1 and 11.

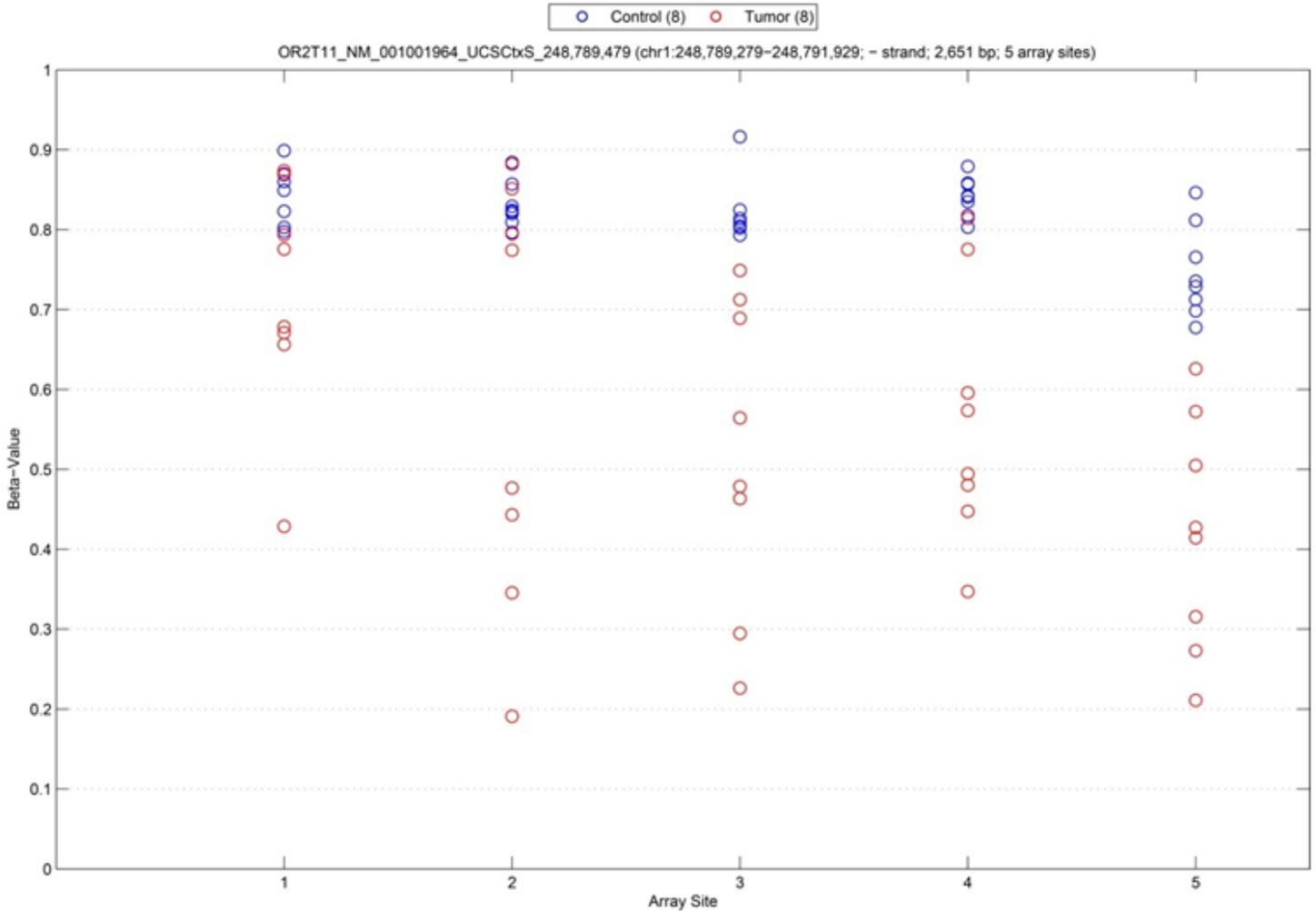


Figure 3

A snapshot of the locations in chromosome 1 (chr1:248,789,279-248,791,929; -strand; 2,651 bp) with example of marked differentially hypomethylated OR genes (OR2T11) in 5 array sites.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Table1SmellIdentification.docx](#)
- [SuppFigures.docx](#)
- [SuppTable1genes.docx](#)
- [SuppTable2.docx](#)