Asymmetric Cancer Hallmarks in Breast Tumors on Different Sides of the Body

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Abstract

During the last decades it has been established that breast cancer arises through the accumulation of genetic and epigenetic alterations in different cancer related genes. These alterations confer the tumor oncogenic abilities, which can be resumed as cancer hallmarks (CH). The purpose of this study was to establish the methylation profile of CpG sites located in cancer genes in breast tumors so as to infer their potential impact on 6 CH: i.e. sustained proliferative signaling, evasion of growth suppressors, resistance to cell death, induction of angiogenesis, genome instability and invasion and metastasis. For 51 breast carcinomas, MS-MLPA derived-methylation profiles of 81 CpG sites were converted into 6 CH profiles. CH profiles distribution was tested by different statistical methods and correlated with clinical-pathological data. Unsupervised Hierarchical Cluster Analysis revealed that CH profiles segregate in two main groups (bootstrapping 90–100%), which correlate with breast laterality (p = 0.05). For validating these observations, gene expression data was obtained by RealTime-PCR in a different cohort of 25 tumors and converted into CH profiles. This analyses confirmed the same clustering and a tendency of association with breast laterality (p = 0.15). In silico analyses on gene expression data from TCGA Breast dataset from left and right breast tumors showed that they differed significantly when data was previously converted into CH profiles (p = 0.033). We show here for the first time, that breast carcinomas arising on different sides of the body present differential cancer traits inferred from methylation and expression profiles. Our results indicate that by converting methylation or expression profiles in terms of Cancer Hallmarks, it would allow to uncover veiled associations with clinical features. These results contribute with a new finding to the better understanding of breast tumor behavior, and can moreover serve as proof of principle for other bilateral cancers like lung, testes or kidney.
Introduction

During the last two decades, it has been established that cancer is in essence a genetic disease. Over the past years, the cancer genome has been studied by different molecular strategies, revealing alterations in many cancer related genes. And several studies have shown that the majority of human tumors carry mutations in a subset of genes, composed of 30 to 60 different affected loci [1]. Inferences drawn from these observations indicate that cancers as diseases, are caused by the emergence of distinct “genomic landscapes” composed of a combination of these mutations. A “cancer genomic landscape” for a particular tumor is known to be composed by few genes called “drivers” (frequently found altered across many cancers), and many more genes called “passengers” (seldom found altered across cancers) [1]. Consequently, these discoveries have guided the development of targeted therapies against single driver genes, including gefitinib and erlotinib for non-small-cell lung cancer patients with EGFR mutations [2], panitumumab and cetuximab for metastatic colon cancer with amplified expression of EGFR [3], vemurafenib for patients with melanomas carrying BRAF mutations [4], and crizotinib for lung cancer patients carrying EML4-ALK translocations [5]. However, in spite of all the remarkable advances, new information strongly suggests that therapeutically targeting single driver genes is not a reliable strategy for the long term treatment of cancers [1,2], since cancer traits appear to be better described when genomic data is interpreted as a network of combined functional pathways. “Cancer genomic landscapes” functioning as interconnected pathways may help to explain the existence of “Cancer Hallmarks” (CH) proposed by Hanahan and Weinberg in 2000 [6]. They propose that the cancer phenotype is characterized by a few biological capabilities acquired during a multistep process of carcinogenesis, i.e. sustained proliferative signaling, evasion of growth suppressors, resistance to cell death, enabled replicative immortality, induction of angiogenesis, and activation of invasion and metastasis. Emerging evidence also indicates that the reprogramming of energy metabolism and evasion of immune destruction could be considered two new cancer hallmarks [7]. These six to eight cancer hallmarks provide an organizing principle for understanding the complexity and diversity of neoplastic diseases.

Moreover, in addition to genetics, epigenetics, a mode of inheritance that is brought about independently of genetic sequences, offers a complementary paradigm for better understanding the behavior of cancer. Epigenomics have revealed that cancers can also be caused and maintained by inherited alterations of gene expression networks. Thus, it has become evident that CHs are acquired by a successive accumulation of both genetic and epigenetic alterations, which are transmitted to subsequent cellular generations. These genetic and epigenetic alterations occur at many different genomic regions [1] but their effect still converges in less than ten tumor capabilities [7], which implies that the gene functions involved in tumorigenesis are function of the state of the mutated network [8]. Since the study of epigenetic alterations lags significantly behind those performed in genetics, in the current study, we sought to better understand the role of epigenetic alterations that occur during breast cancer and unraveling their potential application in the diagnosis and prognosis of this disease. Although many genes have been encountered aberrantly methylated in breast tumors and associated to worst prognosis [9–13], the breast cancer field still lacks information for unifying treatment decisions based on epigenetic markers. Epigenetic alterations in breast cancer consist of chromatin modifications, including DNA methylation and histone modifications, and regulatory small and large RNA molecules, contributing all in different ways to regulate gene expression. The best understood among them is DNA hypermethylation, which occurs within CpG islands (CGIs), (particularly in the promoter and first exon regions), blocking the binding sites of transcription factors and therefore strongly associated with gene repression [14].
The purpose of this research work was to establish the methylation profile of CpG sites located in cancer related genes (mainly tumor suppressor genes and DNA repair genes) in fresh human breast tumors so as to infer their potential impact on Cancer Hallmarks. Our results indicate that by converting methylation profiles in terms of Cancer Hallmarks, it would allow to better interpret this type of epigenetic data in a holistic manner and uncover veiled associations with clinical features. Therefore, the mechanistic importance and biomedical relevance of these results are discussed.

**Materials and Methods**

**Patients and tumor samples**

Patients (113) were enrolled in the current study after obtaining their written informed consent based on the scientific and ethical principles of the World Medical Association’s Declaration of Helsinki (Table 1). Ethical approval of the project and the written informed consent was obtained from the Ethics Committee of the Faculty of Medical Sciences, from the National University of Cuyo, Mendoza, Argentina. One hundred thirteen tumors were collected for methylation analyses, to establish their methylation frequencies in the sample cohort. Seventy-six presented complete clinical-pathological data, of which a cohort of 51 was used for further conversion to CH profiles and posterior statistical analyses and a different cohort of 25 tumors was used to perform Real-Time PCR experiments. We generated a database containing the clinical-pathological information of each patient, in addition to the DNA methylation and gene expression profiles of the respective tumors (S1 File). The clinical-pathological features were assessed by the same pathologist and all the patients were treated in either the Gineco-Mamario Institute or the Italian Hospital of Mendoza, Argentina.

**DNA extraction**

Fresh tissues were frozen at -80°C and broken with a frozen mortar. The homogenate was collected and suspended in T10E buffer (10mM Tris/HCl and 1mM EDTA). All samples were stored for at least 24 hours at -20°C and DNA was collected as previously described [15]. Briefly, homogenate from tumor tissues was suspended in 3ml of Cetyl Trimethylammonium Bromide (CTAB) solution (2g/l CTAB, Sigma Aldrich, Bavaria Germany, 100mM Tris/HCl, 20mM EDTA and 2% 2-mercaptoethanol) and incubated at 60°C during 4 hours for membrane lysis. Afterwards, 3ml of chloroform-isoamylic solution (24:1) was added and centrifuged at 3000 rpm for 5 minutes. Aqueous phase was collected into a new tube and mixed with 9ml ice-cold 100% ethanol. Precipitated DNA was dissolved in T10E buffer and stored at -20°C.

**Methylation profiles determination by Methyl specific-Multiplex Ligation-dependent Probe Amplification assay (MS-MLPA)**

To assess the methylation status of 96 CpG sites located within 54 cancer related genes, the MS-MLPA kits ME001, ME002, ME003 and ME011 were used (MRC-Holland, Amsterdam, The Netherlands, [www.mlpa.com](http://www.mlpa.com)). The MS-MLPA assays were performed basically according to manufacturer’s recommendations, introducing subtle modifications (i.e. extended restriction enzyme incubation time, separated ligation and restriction steps) [12]. The fluorescent-labeled PCR products were separated by capillary electrophoresis in an ABI-3130 sequencer (Applied Biosystems, Foster City, CA, USA) and analyzed by GeneMarker v1.75 software (Softgenetics, State College, PA, USA). This analysis normalizes the data by dividing the peak area of a single probe by the peak areas of control probes. Subsequently, the normalized peaks from the samples were divided by the normalized peaks from controls to obtain the Methylation Lateral Asymmetries in Breast Cancer
Dosage Ratio (MDR). A CpG site was considered to be methylated when the MDR observed between sample and control was superior to the cut-off threshold of 8% [15,16]. Afterwards, DNA methylation data was dichotomized in unmethylated and methylated status.

Gene expression analyses by Real-Time Polymerase Chain Reaction

RNA was extracted from fresh tumors with Trizol Reagent (Life Technologies, USA). Five μg of total RNA was used for first strand synthesis of cDNA by using M-MLV retro-transcriptase (Promega, USA) and Random Hexamers (Roche, USA) primers. The reverse transcription...
reaction was carried out during 60 minutes at 37°C according to manufacturer´s instructions. One hundred ng of cDNA were used to perform Real-Time PCR using specific primers for 32 cancer related genes (Table 2) and GAPDH, TBP and HPRT1 as housekeeping genes in a CFX96 thermocycler (Bio-Rad Laboratories, USA). Detection of PCR product was carried out using the specific DNA dye SYBR Green (Bio-Rad, USA). The amplification program consisted of 45 cycles of 15 seconds at 95°C and 60 seconds at 60°C, followed by a final melting curve step. The specificity of the PCR products was assessed by melting curve analysis. Relative expression normalization of genes of interest was carried out using the housekeeping genes GAPDH, TBP and HPRT1 (gene expression as endogenous reference control by the ddCt method). Cycle Threshold (Ct) and Efficiency (EAmp) values were calculated by means of Bio-Rad CFX Manager 3.0 (Bio-Rad Laboratories, USA). After relativizing gene expression to the housekeeping genes, data of each gene was expressed in percentages considering as 100% the highest expression value among all tumors. Subsequently, values were inverted by applying the equation “100 – expression value”. This was necessary to multiply afterwards the inverted data by the “Translation Matrix” in order to create the CH profile matrix for further clustering analyses (see Results Section).

Statistical analyses
To determine whether the methylations in the included genes were independent events, we generated 1000 hypothetic tumors and compared the distribution of CHs (derived from methylation profiles) between experimental and hypothetical tumors. For this, Kolmogorov-Smirnov test was applied using software KyPlot 3.0. To test tumor distribution based on CH profiles, Unsupervised Hierarchical Cluster Analysis using the software MultiExperiment ViewerMeV v4.6 (TM 4 group, Dana Farber Cancer Institute, Boston, MA, USA) was used. For this last analysis, we considered 90–100% bootstrapped clusters. To asses which CH contributed to the clustering, Principal Component Analyses (PCA) and Multiple Regression Analyses of clusters vs. CH were performed applying software InfoStat v.2014 (Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina). For association studies between clinical-pathological variables and clusters, Simple Regression Analyses were performed using the software SPSS v17 (SPSS Inc, Chicago, IL, USA). Multiple Regression Analyses on partitioned samples were performed to determine associations between CH and clinical-pathological variables by the software InfoStat v.2014 (Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina).

Results
Conversion of MLPA-derived Methylation Data into Functional Cancer Hallmarks
We applied the Methyl Specific Multiplex Ligation-dependent Probe Amplification assay (MS-MLPA) to determine the methylation status of 96 CpG sites located within 54 cancer related genes of 113 breast carcinomas. It is important to take in account that previous studies of our group revealed that none of these CpG sites is methylated in normal breast tissue obtained from surgical margins or in circulating leucocytes [12,15]. Therefore, it becomes reasonable to only focus on aberrant methylation events since interference of normal cells is discarded. The methylation frequency of each CpG site was determined on the complete tumor cohort. Based on the obtained frequency, in order to decrease the ambiguity given by the inclusion of poorly methylated sites, we excluded CpG sites methylated in less than 10% of the samples. This criterion reduced the number of CpG sites from 96 to 81, located within the
Table 2. CpG location, genes and methylation frequencies in 113 breast tumors.

| CpG sites respect to ATG | Gene Name (HGDB) | Chromosome Location | Methylation Frequency (%) | CpG sites respect to ATG | Gene Name (HGDB) | Chromosome Location | Methylation Frequency (%) |
|-------------------------|------------------|---------------------|--------------------------|-------------------------|------------------|---------------------|--------------------------|
| 72 bp to exon 2         | APC              | 5q22                | 43.68                     | 13 bp before            | MLH1            | 3p22.2              | 4.42                     |
| 4457 bp before ATM      | 11q23            | 3.6                 | 206 bp after              | MLH1                    | 3p22.2          | 7.69                |
| 4658 bp before ATM      | 11q23            | 4.6                 | 85–86 bp in               | MLH3                    | 14q24.3         | 1.54                |
| 870 bp before BCL2      | 18q21.3          | 4.42                | 269 bp before             | MSH2                    | 14q24.3         | 8.85                |
| 1211 bp before BRCA1    | 17q21.31         | 10.34               | 193 bp before             | MSH2                    | 14q24.3         | 8.85                |
| 1321 bp before BRCA1    | 17q21.3          | 7.21                | 124 bp after              | MSH2                    | 2p21            | 8.85                |
| 852 bp before BRCA2     | 13q12.3          | 6.9                 | 485 bp before             | MSH3                    | 5q14.1          | 35.38               |
| 771 bp before BRCA2     | 13q12.3          | 9.73                | 317 bp before             | MSH6                    | 2p16.3          | 5.75                |
| 157 bp after            | CALCNA1A         | 19p13.2             | 31.53                     | MSH6                    | 2p16.3          | 1.54                |
| 32 bp before            | CALCNA1G         | 17q21.33            | 8.18                      | MSH6                    | 2p16.3          | 7.69                |
| 8560 bp before          | CASP8            | 2q33.2              | 8.14                      | MSH6                    | 11p13           | 60.92               |
| 1168 bp before          | CCND2            | 12p13.3             | 72.41                     | MSH6                    | 11p13           | 41.38               |
| 1358 bp before          | CCND2            | 12p13.3             | 54.02                     | MSH6                    | 7p22.1          | 9.91                |
| 17 bp before            | CD44             | 11p12               | 18.92                     | MSH6                    | 7p22.1          | 12.31               |
| 411 bp before           | CD44             | 11p12               | 15.04                     | MSH6                    | 7p22.1          | 3.19                |
| 42 bp before            | CDH13            | 16q23.3             | 28.32                     | MSH6                    | 10q23.3         | 4.42                |
| 157 bp before           | CDKN1B           | 12p13.2             | 3.08                      | MSH6                    | 10q23.3         | 12.61               |
| 997 bp before           | CDKN2A           | 9p21                | 0.9                       | MSH6                    | 3p24.2          | 16.22               |
| 31 bp after             | CDKN2A           | 9p21.3              | 7.21                      | MSH6                    | 3p24.2          | 26.44               |
| 110 bp before           | CDKN2B           | 9p21                | 17.12                     | MSH6                    | 3p24.2          | 6.05                |
| 714 bp before           | DAPK1            | 9q22                | 18.02                     | MSH6                    | 3p24.2          | 67.57               |
| 366437 bp after         | DLC1             | 8p22                | 20.69                     | MSH6                    | 3p24.2          | 72.97               |
| 366993 bp after         | DLC1             | 8p22                | 6.19                      | RB                      | 13q14.2         | 6.15                |
| 163 bp after            | ESR1             | 6q25.1              | 20.72                     | RB1                     | 13q14.2         | 11.49               |
| 658 bp before           | GATA5            | 20q13.3             | 21.84                     | RUNX3                   | 1p36.11         | 24.78               |
| 103 bp after            | GSTP1            | 11q13               | 29.73                     | SCGB3A1                 | 9q35            | 66.67               |
| 245 bp after            | GSTP1            | 11q13               | 14.16                     | SCGB3A1                 | 9q35            | 38.05               |
| 953 bp before           | ID4              | 6p22.3              | 57.52                     | SFRP4                   | 7p14.1          | 20.69               |
| 319 bp before           | ID4              | 6p22.3              | 27.59                     | SFRP5                   | 7p14.1          | 21.05               |
| 305 bp before           | IGSF4            | 11q23               | 16.22                     | SFRP5                   | 10q24.1         | 0.71                |
| 72 bp before            | IGSF4            | 11q23               | 32.18                     | SFRP6                   | 10q24.1         | 18.6                |
| 432 bp before           | MGMT             | 10q26.3             | 3.08                      | SFRP5                   | 10q24.1         | 18.6                |
| 346 bp before           | MGMT             | 10q26.3             | 3.08                      | THBS1                   | 15q15           | 9.2                 |
| 93 bp before            | MGMT             | 10q26.3             | 20.35                     | TIMP3                   | 22q12.3         | 13.51               |
| 151 bp before           | MGMT             | 10q26.3             | 3.08                      | TIMP3                   | 22q12.3         | 4.42                |
| 382 bp before           | MGMT             | 10q26.3             | 1.54                      | TIMP3                   | 22q12.3         | 8.11                |
| 233 bp after            | MLH1             | 3p22.3              | 0                         | TP53                    | 17p13.1         | 6.19                |
| 659 bp before           | MLH1             | 3p22.3              | 1.8                       | TP73                    | 1p36.32         | 2.7                 |
| 518 bp before           | MLH1             | 3p22.1              | 0                         | 29551 bp before         | TP73            | 45.05               |
| 383 bp before           | MLH1             | 3p22.2              | 1.77                      | 412 bp before           | TP73            | 10.77               |
| 246 bp before           | MLH1             | 3p22.1              | 12.31                     | TWIST1                  | 11p13           | 89.38               |

doi:10.1371/journal.pone.0157416.t002
promoter of 43 distinct cancer related genes (Table 2). Thus, the 113 tumors served to determine methylation frequencies and establish the 43 crucial genes to continue the study.

Subsequently, the conversion of methylation data into CH profiles had to be performed. We decided to perform this only for 51 tumors of which we had complete clinical-pathological information. For this purpose, we organized the methylation data of the 51 tumors into a matrix which included for each tumor, the methylation status of each CpG site, determined by a dichotomized criterion (unmethylated and methylated).

For the conversion into cancer hallmarks, we selected 5 of the 6 cancer hallmarks proposed by Hanahan and Weinberg, i.e. sustained proliferative signaling (CH1), evasion of growth suppressors (CH2), resistance to cell death (CH3), induction of angiogenesis (CH4) as well as activation of invasion and metastasis (CH6). We did not include the Hanahan-Weinberg hallmark “enabled replicative immortality” because our study lacked of genes related to this feature. Instead, we decided to consider the alternative hallmark “genome instability” (CH5) (Fig 1), given many included genes (15 of 43) had a role in DNA repair.

By data mining studies, including literature and databases from NCBI (http://www.ncbi.nlm.nih.gov/), Uniprot (http://www.uniprot.org/), Nextprot (http://www.nextprot.org/) and DAVID Bioinformatics Resources 6.7 (https://david.ncifcrf.gov/), the influence of the 43 genes on the 6 selected CH was defined. For this purpose, we developed a priori a gene-to-function Participation Index (PI), with values ranking from 0 (no participation) to 3 (high participation), subsequently adjusted by a correction coefficient (ranked from 0 to 1) depending on data reliability, potentially contradictory data, or ambiguous interpretation to derive an Adjusted Participation Index (API). It is worth to remember that the participation of a gene in a specific CH was inferred considering the scenario in which the gene was methylated. These efforts led us to establish, that among the 43 genes, 24 of them associated to functions compatible with CH1, 7 genes in CH2, 20 genes to CH3, 15 genes to CH4, 16 genes to CH5 and 16 genes to CH6. Thus, from this data we inferred that all the CHs were related to at least 7 genes and that a single gene could be included in more than one CH. So, the API values were established for the 43 genes, generating what we called the “translation matrix”.

Now, by using this “translation matrix” the conversion of methylation data into the CH profiles had to be performed. By multiplying the methylation data and the “translation matrix”, we derived a CH Matrix (CHM) (Fig 2A), which shows the tumor traits now at a defined set of Hallmarks level. The higher the CHM values obtained, the stronger the magnitude of methylated derived cancer features. This data modeling approach considers that a given CH was supposed to be acquired if at least one of its component genes was methylated. Besides, some genes are members of multiple CHs, e.g. TIMP3 whose methylation is associated with CH4 and CH6, or ATM which is enrolled in CH1, CH3, CH5 and CH6 (S1 Fig). If such a gene was methylated, all hallmarks in which the gene had participation were considered to be enhanced.

Fig 2B shows the results obtained by converting MLPA-derived methylation data of 51 tumors into CH profiles. At first sight it can be observed that most of the tumors displayed CH1 (sustained proliferative signaling) and CH3 (resistance to cell death) as their strongest capabilities.

**Statistical Evidence Supports a Non Random Distribution of CH profiles in Breast Tumors**

From Fig 2B, we could conclude that the tumors were not similar. We speculated that CpG methylations are not independent events but rather are probably coordinated to affect the CH capabilities of the tumors in specific manners. To test these possibilities, we generated 1000 hypothetic tumors incorporating methylations of cancer related genes with a probability equal...
to the experimental methylation frequency. The purpose was to compare the observations of CHs in fresh (experimental) tumors, with randomly acquired CHs in artificial (hypothetical) tumors. For example, suppose the CpG site 1 presented a 15% frequency of methylation among experimental tumors. To create a hypothetical tumor, the methylation status of CpG site 1 was decided by generating a random number between 0 and 1 and considering the site methylated when the number was less than 0.15. This was performed for each of the 81 CpGs (considering the methylation frequency of each site) in 1000 artificial tumors. Afterwards, the methylation profiles were converted into CH profiles. With the CH values obtained by experimental and artificial tumors, we generated frequency histograms of the 6 CHs and compared whether they presented differences in a single or global way. We detected a significant difference for the global CH index (which resulted by summing the normalized single CH of each tumor) (Fig 3A) and for CH1 and CH6 (Fig 3B). These differences were suggesting that the CH profiles in experimental tumors were not generated by random accumulation of CpG methylations. If so, we speculated that the experimental tumors could be grouped depending on the CH acquisition. To test this, we performed Unsupervised Hierarchical Cluster Analyses of the CH profiles for the 51 experimental tumors.

The test gave rise to two significant groups, whose robustness was determined by a 90–100% bootstrapped confidence interval (based on 100 iterations, average linkage and Manhattan distance metric) (Fig 4A). This was revealing that the tumors tended to cluster based on...
Fig 2. Translation of Methylation profile to Cancer Hallmark profile. (A) The scheme describes how the MLPA-derived methylation data was converted into CH profiles, though a translation matrix. A multiplication operation was performed on two matrices: the Methylation Profile Matrix (MPM) and the Translation Matrix (TM). The MPM holds information of 51 CpGs located in 43 genes the MPM, for 51 tumors with complete clinical-pathological information. Green boxes represent the un-methylated status and red boxes the methylated status. The TM contains the Adjusted Participation Index (API) which expresses in a rank from 0.
CH acquisition, which was in line with the fact that the acquisition was not at random. In order to understand more deeply how this grouping was occurring, we decided to investigate whether any of the 6 CHs had a main role in the group formation, so we performed a Principal Component Analysis (PCA) of the data. This analysis calculates the power of each CH (or Component) to predict the groups; the higher the power, the more principal is the CH for the group formation. This test showed that the clustering could be predicted in 92.7% of cases by CH1 and CH6 (Fig 4B). Another way of testing prediction value of the CHs is by Multiple linear Regression Analysis of clusters vs. CHs. This test confirmed that CH1 (p < 0.01), CH4 (p = 0.05) and CH6 (p = 0.11) were the best predictors of this grouping (adjusted R² = 0.72).

On the contrary, artificial tumors did not reveal the same behavior, as PCA analyses performed on artificial tumors showed statistically different values (76.9% prediction power for CH1 and CH6) as compared with the experimental tumors. So, taken all together we were seeing that the experimental tumors were tending to cluster in two groups based on their CH profiles and that CH1 and CH6 were principal actors in this differentiation.

Interestingly, this was observable only at a CH level and not at methylation level data. When we performed Unsupervised Hierarchical Cluster Analysis on methylation profiles (instead of CH profiles), no clusters for 90–100% bootstrapped confidence intervals did appear. This was confirming that the conversion of MLPA-derived methylation data into functional CH profiles had been worth since analyses at hallmark-levels were revealing more information about the tumors.

Cancer Hallmarks Profiles of Experimental Tumors More Robustly Associate to the Single Clinical-Pathological Feature of Breast Laterality (BL)

The next question which arose was: do the two tumor groups generated by CH profiles share clinical features? To address this question, we evaluated the association between the clusters and the clinical-pathological variables listed in Table 1, i.e. age, axillar lymph node status, tumor stage, disease stage, breast laterality and molecular subtype. Surprisingly, by Regression Analysis, only the variable breast laterality (BL) showed significant association with the tumor groups (p = 0.05). Remarkably, this was not observable when testing correlation between single methylation profiles with BL, supporting again the concept that hallmark-level analyses were more informative. So, the association of CH profiles with BL was suggesting us the hypothesis that tumors from different breast sides presented distinctive cancer hallmarks (breast laterality hypothesis, BLH).

We were aware that the general understanding is that the breast tumor would be randomly located and would present similar features independently from the side. Consequently, to deepen the analysis, we partitioned the data in left and right and performed Multiple Regression tests by correlating the tumors with both the CHs and clinical-pathological variables. Indeed, in support of the laterality hypothesis stated above, this approach showed that the results differed according to breast side. In left sided lesions, CHs were better predictors of the tumor stage (adjusted R² = 0.76), revealing that CH1 increased with tumor stage while CH4,
Fig 3. Cancer Hallmark profiles are not randomly distributed in experimental tumors. Histograms presenting the distribution of CH values in experimental vs artificial (hypothetical) tumors. (A) By summing the values of the 6 CHs in each tumor, a Global CH value was obtained. Comparisons of the histograms representing the distribution of the Global CH values of experimental vs hypothetical tumors revealed a significant difference (Kolmogorov Smirnov test (KS*), p<0.05). (B) By performing the comparison of the distribution of the single CH values of experimental vs hypothetical tumors, a significant difference was detected for CH1 and CH6 (Kolmogorov Smirnov test (KS*), p<0.05).
These differences are suggesting that the CH profiles in experimental tumors are not generated by random accumulation of CpG methylations.

CH5 and CH6 decreased (Fig 5A). This was not observable in right sided tumors, where the adjusted R² was 0.00 for tumor stage (Fig 5B), supporting thereby the conclusion that the behavior of CHs is different in tumors of different sides.

We expanded our analyses in order to assess the possibility of confounding factors. In order to discard any influence of interfering variables, we compared the number of tumor types, axillary lymph nodes status, tumor grades, disease stages and receptor expression levels in left vs right tumors and detected no significant differences. By this we were able to exclude any interference of other clinical-pathological variables. Therefore, based on these evidences, we are able to propose that CHs inferred from methylation data are acquired in distinct ways according to the breast side on which the tumor develops.

Expression Profiles Also Associate to the Single Clinical-Pathological Feature of Breast Laterality

To continue challenging our BL hypothesis, we thought to test a new cohort of tumors from a different perspective. We decided to validate the results thus far generated through the analysis of gene expression levels instead of methylation statuses, in a different tumor cohort (Table 1). The rationality of this was that even though not all methylation events included in our study would have the same effect on gene expression, if the associations inferred from methylation data were robust, we would be able to detect them also inferring from expression data. For this purpose, we performed Real-Time PCR analyses on 32 (Table 3) of the 43 genes on a new
cohort of 25 breast carcinomas. The expression data was normalized to the average of 3 housekeeping genes and organized in an expression matrix which contained the information of the 25 new tumors. In a manner which is similar to the approach used above to convert methylation into CH level information, we converted now the expression matrix into CH profiles, through the "translation matrix" (shown in Fig 2A). Accordingly, we obtained a CH profile matrix, which allows drawing inferences from expression information rather than from methylation profiles. Notice that in contrast to the methylation results, the expression data appears to be inversely related to the CH profiles. Thereby, we inverted the values for further clustering analyses.

When assessing whether the latter CH profiles -now inferred from expression profiles- segregated in a non-random manner, again we detected the formation of two groups by Unsupervised Hierarchical Cluster Analysis using a 90–100% bootstrap. The formation of these two groups encouraged us to perform further association analysis of the clusters with clinical variables. For subsequent Regression Analyses, p values less than 0.15 were considered relevant, and based on these criteria a unique association was observed between clusters and the clinical variable BL (p = 0.12) (Fig 6A). Again, when testing which CH had main participation in the cluster formation, multiple lineal regression analysis of clusters vs. CHs revealed that CH2, CH5 and CH6 were predictors of this clustering event (adjusted R² = 0.67). When we checked whether this was observable when analyzing expression profiles (not converted to CH profiles), no clusters with 90–100% bootstrap were observed.

So far, experimental data inferred from methylation and expression analyses in different tumor cohorts were sustaining our BL hypothesis. To deeper confirm this, we searched for an in silico validation, by analyzing the public dataset from Oncomine Research Edition Platform (gene expression signatures). Filters were set for invasive breast cancer from the dataset “TCGA Breast”. Two hundred fourteen tumors presented anatomic location information (115 left sided and 99 right sided) and 28 of the 32 genes presented mean expression data (excluding RARB, DAPK1, MSH2 and DLC1 of Table 3). The expression data were similarly analyzed as data from experimental results: we inverted the expression mean values and converted them with the "translation matrix" to obtain a CH profile matrix. By unpaired Student test, we tested the means of left vs right sided tumors and surprisingly a significant difference was observed among them (p = 0.033, t = 2.45, R² = 0.37).

Fig 5. Tumors from left-right breast sides are differentially predicted by cancer hallmarks. Regression analyses of CHs vs tumor stage in left and right sided tumors. (A) CHs of left sided lesions were better predictors of the tumor stage, revealing that CH1 increased with tumor stage while CH4, CH5 and CH6 decreased (adjusted R² = 0.76). (B) CHs of right sided tumors have no predictable value for tumor stages (adjusted R² = 0.00), supporting thereby the conclusion that the behavior of CHs differs in tumors of different sides.

doi:10.1371/journal.pone.0157416.g005
Thus, these results validated our previous methylation-based observations in two manners: first, by detecting experimentally the same association between clusters and breast sides at a gene-expression level in a new cohort of fresh tumors; and secondly, by detecting the same difference in silico, when converting expression data of public databases into CH profiles.

**Discussion**

Tumors evolve from benign to malignant cells by acquiring consecutive mutations over time. Similar to an evolution process which is modulated by natural selection pressures, during the tumorigenesis process the tumor acquires traits that confer survival advantages to the cells. Thereby, the acquired mutations which are found in the final tumor should have passed a selection filter and many of them are contributing in different levels to certain cancer pathways. Because most cellular processes involve multiple proteins functioning in a concerted and redundant manner, it is possible that mutations in different genes result in similar tumorigenic

### Table 3. Genes included in the expression analyses.

| Gene Name (HGDB) | Chromosome Location |
|------------------|---------------------|
| APC              | 5q22                |
| ATM              | 11q23               |
| BRCA1            | 17q21.3             |
| BRCA2            | 13q12.3             |
| CCND2            | 12p13.3             |
| CD44             | 11p12               |
| CDH13            | 16q23.3             |
| CDKN1B           | 12p13.2             |
| CDKN2A           | 9p21                |
| CDKN2B           | 9p21                |
| DAPK1            | 9q22                |
| DLC1             | 8p22                |
| ESR1             | 6q25.1              |
| GSTP1            | 11q13               |
| ID4              | 6p22.3              |
| MGMT             | 10q26.3             |
| MLH1             | 3p22.3              |
| MSH2             | 2p21                |
| MSH6             | 2p16.3              |
| PAX6             | 11p13               |
| PMS2             | 7p22.1              |
| PTEN             | 10q23.3             |
| RARB             | 3p24.2              |
| RASSF1           | 3p21.3              |
| RB1              | 13q14.2             |
| SCGB3A1          | 5q35                |
| SFRP4            | 7p14.1              |
| THBS1            | 15q15               |
| TP53             | 17p13.1             |
| TP73             | 1p36.32             |
| TWIST1           | 7p21.2              |
| WT1              | 11p13               |

doi:10.1371/journal.pone.0157416.t003
effects [17]. Thereby, when performing single-gene mutation studies, the complete tumor features can be missed. In contrast, when performing whole-genome studies the overwhelming data can disturb the identification of specific tumor traits. When we clustered the studied genes in 6 selected cancer hallmarks and performed the statistical analyses on a higher organization level, we were able to notice the different hallmarks of right and left sided breast tumors. In the current study we show for the first time, that breast carcinomas arising on different sides of the body present differential cancer traits inferred from methylation profiles. The validation by the expression analyses performed on a new experimental cohort of carcinomas and on public databases information supports our conclusions. Notably, numerous previous studies using single gene markers have failed in establishing a correlation between laterality and either methylation or expression levels. The fact that right and left sided breast tumors acquire differential tumor traits is reflected by our approach through the mean of transforming genetic and epigenetic data into cancer hallmarks.

Since the general understanding is that the breast tumors of different sides do not differ in behavior, clinical features, treatment response, etc., it becomes important to make clear whether no statistical artefacts are interfering in our results. A statistical artefact results from a bias in the collection, manipulation and/or measurement of the data. In our study, we can demonstrate that the bias sources have been avoided as discussed further on. The collection biases can be discarded since analyses were performed on two different and independent cohorts of breast tumors. The laterality of tumors in both cohorts was similar (left/right percentages: 56%/44% and 52%/48%, CI: 0.23–2.9, p = 0.01) and both cohorts revealed association of the cancer hallmark profiles with laterality. The manipulation biases can also be discarded based on the fact that the results have been observed on assays performed on two different molecules: DNA and RNA. That the observations based on the methylation assays (performed on DNA) and on the RealTime PCR expression assays (performed on RNA) converged into a similar association with side, shows that no manipulation of samples could be generating an artefact in
the results. Furthermore, *measurement biases* can produce partial perceptions due to the used methods. We are aware of the potential artefacts that can appear when statistics is applied as a single approach. We therefore proposed questions which required different statistical analyses and who’s results converged and were in line with the same concept: cancer hallmark profiles differ among breast tumors of different sides. To answer the question whether tumors were distributed at random based on the cancer hallmark profiles, Unsupervised Hierarchical Cluster Analyses were performed. The formation of 2 significant clusters was observed both, from DNA methylation inferred data as from RNA expression analyses. It is worth to remark that the RNA expression observations were obtained in a complete different tumor cohort. To answer the question whether one specific cancer hallmark was contributing to the grouping of tumors, two different statistical analyses were applied: PCA and Multiple Regression Analyses. Both revealed that only some of the cancer hallmarks were relevant for the generation of the clusters. To answer if a clinical-pathological variables was associated with the clusters Simple Regression Analyses were performed and again, methylation and expression inferred data revealed association with side. Another way to confirm this association, was to analyze it backwards: partitioning the tumor sample in left and right sided, and by detecting a different distribution of the cancer hallmarks.

Taken all this together, we can asseverate that the observations are not statistical artefacts, in which case at least some of all the approaches (change of tumor cohort, change of molecule, change of statistic method) should have invalidated the rest of the observations.

And finally, the TCGA data support our findings, in an enhanced population of breast tumors (214 invasive ductal carcinomas). This has, to our consideration, the strongest statistical power to confirm and validate our results.

Among the different statistical analyses, CH6 (Invasion and Metastasis) appears as a key hallmark related to BL. Sixteen of the 43 studied genes do have a role in CH6, i.e. TIMP3, APC, ATM, PTEN, CD44, RASSF1A, CDH13, TP53, PAX5, THBS1, GATA5, DLC1, SFRP5, SFRP4, TWIST1 and RUNX3. The results evidence that in left sided tumors the CHM values for CH6 are higher than in right sided tumors. This challenging observation is difficult to interpret in clinical terms since deeper studies should evaluate the functional impact of high CH6 values on tumor cells behavior. We consider the consistent results on CH6, however, as a powerful support to postulate this cancer hallmark as the icon on which the BLH can be visualized.

Even though several studies present apparently discordances between laterality associations with clinical variables [18,19,20,21,22] Veltmaat et al. integrate these data in their recent review and discuss very interestingly that left-sided tumors are associated with more affected nodes despite the left-right asymmetry in overall node number. Therefore, metastasis to lymph nodes is proposed by them to be a lateralized disease feature, and may indicate left-right differences in tumor biology, e.g. greater metastatic potential of left-sided tumors and cancer, which is line with our observations.

Even though apparently symmetric, left and right sides of vertebrates’ bodies are different. Internal bilateral organs such as breast glands present differences in volume, structure and position. In tumorigenesis of paired organs, laterality has been described in breast [23], lung, testes [24] and kidney cancer [25]. Higher incidence of lung, ovarian and testicular cancer is found on the right side, whereas melanoma and breast cancer is approximately 10% and 5% respectively more likely to be diagnosed in the left side [26,27]. Speculations have been proposed to explain these diverse incidences, based on different organ sizes, handedness [28] and sun exposure [29]. However, the underlying reasons remain unknown.

In colon cancer, some works have discovered a tendency of different methylation profiles among left vs right sided adenomas [30,31]. Even though left and right sides of the colon
present much more clear differences in their function and tissue environment, it is a proof of principle in line with our observations in the breast.

Only few studies exist about breast cancer laterality related to tumor behavior. Dane et al. informed in 2008 about an association of breast cancer laterality and lymph node metastasis in human patients [21]. Fatima et al. found that right sided breast tumors were associated with younger age and hormone receptor negativity [32]. Recently, Onibokun et al. informed that left sided predominance was significantly greater for high grade tumors and for hormone receptor negative tumors [33]. It should be interesting to analyze if and how the increment of the left/right ratio in higher grade tumors could be linked to our observations about the better predictive capacity of CH profiles in higher grade left tumors. Still, the regulation of these processes is far from clear.

Left-right sided asymmetries have been informed in gene expression during embryogenesis of the breast development [34]. Several growth factors such as Nodal, Lefty, FGF, HB-EGF and HGF as well as transcription factors (e.g. Pitx2, FoxA2) are considered candidates with overlapping functions in cancer and development laterality [34]. Recently, Robichaux et al. found that mammary glands in wild-type mice have differences in gene expression, and that these differences confer differential susceptibility to HER2-mediated effects on ductal epithelial growth and differentiation [35]. As communicated by Veltmaat et al. based on their observations in mice, it is becoming clear that each mammary gland has an individual identity since left-right asymmetries are acquired during embryonic mammary development [22]. Very interestingly, this latter publication reviews breast cancer left-right asymmetries, however some of them presenting contrasting data. Borisenkov et al. informed higher survival rates in right sided breast tumors of Russian patients [18], and on the contrary, Harveit et al. observed the same in left sided breast tumors of Norwegian patients [19].

Epigenetic alterations are proposed to occur linked to environmental variables [36]. Macro and micro-environment components can induce alterations in the regulated crosstalk which exists between DNA and histone marks, rendering a change in the gene transcriptome [37,38]. The epigenome, in contrast to the genome, is flexible, dynamic and reversible, being thereby a better candidate to respond faster to the environment. In this line, the breast gland is the specific micro-environment in which breast tumors develop. Tumor cells do not act in isolation, but rather subsist in a rich niche provided by resident fibroblasts, leukocytes, and extra-cellular matrix. Thereby, the tumor stroma is an integral part of cancer initiation, growth and progression. Our proposed breast laterality hypothesis sustains that left-right breast gland niches differ since their development. An example could be based on their different distance to the heart resulting in a distinct blood flow. The important role of inflammatory signaling in breast cancer suggests that a gradient in blood stream may influence tumor behavior [22]. We therefore propose that these left-right different micro-environments are the starting elements that during a tumorigenic process provoke distinct epigenetic signatures that are later on transformed into differential cancer hallmarks. We are aware that besides aberrant methylation, other alterations occur during tumorigenesis which can also contribute to the cancer hallmarks. Even though we have not evaluated what happens with the rest of genomic alterations in left and right breast tumors, we can affirm that at least the epigenetic contribution to cancer hallmarks, differs among sides. We are aware that our observations are solid but however partial, since we have included a selection of a few cancer related genes and organized them in a selection of several cancer hallmarks. Therefore, even though we were able to observe the differences among left and right sided tumors, further and deeper studies encompassing an extended genome analysis will give light to the laterality hypothesis.

A limitation of our study is that while in most of the cases it is true that methylation reduces the expression of the involved genes, there exist situations where this simple interpretation
fails. In this study we performed methylation analyses on CpG sites located on promoters and
first exons of cancer related genes, however this does not guarantee that they all regulate gene
expression. It could be possible that in some cases more CpG sites are required for gene silenc-
ing, or where additional alterations, such as a complex crosstalk with histone modifications are
needed for expression regulation. Moreover, genetic alterations such as copy number vari-
tions, translocations and point mutations can co-exist with epigenetic modifications. These
effects have been underestimated in our work and could explain some discrepancies between
methylation and expression data at a single-gene level. However, the impact of this limitation
is reduced, given that both CH profiles -inferred from methylation and expression data- con-
erged on the same association with BL. Therefore, gene expression data became interesting
and powerful to re-evaluate the BLH. Our observations on CH profiles obtained from gene
expression data are a strong support for the hypothesis given that: firstly, the experiments were
performed on a completely new cohort of tumors; secondly, even though methylation events
do not always imply gene silencing (as mentioned above), the laterality association of CHs per-
sisted and raised from a complete different biologic observation. We consider this as a strong
and powerful support for the BLH. Interestingly, the in silico analyses of gene expression signa-
tures validated our experimental observations.

Our conclusions are relevant to the interpretation of breast cancer disease. We have demon-
strated that epigenetic events can describe differential behavior of carcinomas in different
breast sides, which should be informative for designing and better interpreting the results of
experimental therapies. In fact, we propose that the use of similar approaches to the ones
described in the current study may be useful for better understanding the potential link
between heterogeneity in breast laterality with the malignant behavior and prognosis of breast
cancer. Moreover, these results may guide the future design and execution of mechanistic
experiments aimed at defining the genetic and epigenetic basis of side differences in breast can-
cer. Lastly, these results contribute with a new finding to the better understanding of breast
tumor behavior, and can moreover serve as proof of principle for other bilateral cancers like
lung, testes or kidney. In summary, our conclusions may influence future experimentations as
well as diagnostic and therapeutic interventions in cancer types with left-right asymmetries.

Supporting Information

S1 Fig. Complete Translation Matrix. The Figure shows the gene-to-function Adjusted Par-
ticipation Index (API) in a Translation Matrix, with values ranking their participation in the 6
CHs from 0 (no participation) to 3 (high participation). The matrix shows that among the 43
genes considered for our dataset, 24 presented associations to functions compatible with CH1,
7 with CH2, 20 with CH3, 8 with CH4, 15 with CH5, and 16 genes with CH6.
(TIF)

S1 File. Supporting information. The file contains the tumor methylation profiles, the CH-
methylation profiles and the clinical-pathological features of the cohort of 51 tumors and the
tumor expression profiles, the CH-expression profiles and the clinical-pathological features of
the different cohort of 25 tumors.
(XLSX)

Acknowledgments

This work has been in part supported by a Grant from the Argentine National Institute of Can-
cer (INC) and from the Scientific and Technical Secretary (SECTyP) of the National University
of Cuyo, Argentina. EC obtained a fellowship from the American Society of Biochemist and Molecular Biology to perform the gene expression assays.

Author Contributions
Conceived and designed the experiments: LM MR. Performed the experiments: EC SRL MTB GU AM. Analyzed the data: EC LM RU MR. Contributed reagents/materials/analysis tools: FG JO. Wrote the paper: EC LM RU MR.

References
1. Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz LA Jr, Kinzler KW. Cancer Genome Landscapes. Science (80-). 2013; 339: 1546–1558. doi: 10.1126/science.1235122
2. Sequist LV, Martins RG, Spigel D, Grunberg SM, Spira A, Janne PA, et al. First-line gefitinib in patients with advanced non-small-cell lung cancer harboring somatic EGFR mutations. J Clin Oncol. 2008; 26: 2442–2449. doi: 10.1200/JCO.2007.14.8494 PMID: 18458038
3. Amado RG, Wolf M, Peeters M, Van Cutsem E, Siena S, Freeman DJ, et al. Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. J Clin Oncol. 2008; 26: 1626–1634. doi: 10.1200/JCO.2007.14.7116 PMID: 18316791
4. Chapman PB, Hauschild A, Robert C, Haanen JB, Ascierto P, Larkin J, et al. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. N Engl J Med. 2011; 364: 2507–16. doi: 10.1056/NEJMoa1103782 PMID: 21639808
5. Kwak EL, Bang Y-J, Camidge DR, Shaw AT, Solomon B, Maki RG, et al. Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. N Engl J Med. 2010; 363: 1693–703. doi: 10.1056/NEJMoa1006448 PMID: 20979469
6. Hanahan D. The Hallmarks of Cancer [Internet]. Cell. 2000. pp. 57–70.
7. Hanahan D, Weinberg RA. Hallmarks of cancer: The next generation. Cell. 2011. pp. 646–674. doi: 10.1016/j.cell.2011.02.013 PMID: 21376230
8. Sodir NM, Evan GI. Finding cancer’s weakest link. Oncotarget. 2011; 2: 1307–13. 396 [pii] PMID: 22202195
9. Bediaga NG, Acha-Sagredo A, Guerra I, Viguri A, Albaina C, Ruiz Diaz I, et al. DNA methylation epigenotypes in breast cancer molecular subtypes. Breast Cancer Res. 2010; 12: R77. doi: 10.1186/bcr2721 PMID: 20920229
10. Branham MT, Marzese DM, Laurito SR, Gago FE, Orozco JI, Tello OM, et al. Methylation profile of triple-negative breast carcinomas. Oncogenesis. 2012; 1:e17. doi: 10.1088/onscics12.17 PMID: 23552734
11. Dammann R, Yang G, Pfeifer GP. Hypermethylation of the CpG island of Ras association domain family 1A (RASSF1A), a putative tumor suppressor gene from the 3p21.3 Locus, occurs in a large percentage of human breast cancers. Cancer Res. 2001; 61: 3105–3109. PMID: 11306494
12. Marzese DM, Hoon DSB, Chong KK, Gago FE, Orozco JI, Tello OM, et al. DNA methylation index and methylation profile of invasive ductal breast tumors. J Mol Diagnostics. 2012; 14: 613–622. doi: 10.1016/j.jmoldx.2012.07.001
13. Twelves D, Nerurkar A, Osin P, Dexter T, Ward A, Gui GPH, et al. DNA promoter hypermethylation profiles in breast duct fluid. Breast Cancer Res Treat. 2013; 139: 341–350. doi: 10.1007/s10549-013-2544-8 PMID: 23674191
14. Maunakea AK, Chepelev I, Zhao K. Epigenome mapping in normal and diseased states. Circ Res. 2010; 107: 327–339. doi: 10.1161/CIRCRESAHA.110.222463 PMID: 20669072
15. Marzese DM, Gago FE, Vargas-Roig LM, Roqué M. Simultaneous analysis of the methylation profile of 26 cancer related regions in invasive breast carcinomas by MS-MLPA and dMS-MLPA. Mol Cell Probes. Elsevier Ltd; 2010; 24: 271–280. doi: 10.1016/j.mcp.2010.05.002
16. Moelans CB, Verschuur-Maes AHJ, Van Diest PJ. Frequent promoter hypermethylation of BRCA2, CDH13, MSH6, PAX5, PAX6 and WT1 in ductal carcinoma in situ and invasive breast cancer. J Pathol. 2011; 225: 222–231. doi: 10.1002/path.2930 PMID: 21710692
17. Jones S, Zhang X, Parsons DW, Lin JC-H, Leary RJ, Angenendt P, et al. Core Signaling Pathways in Human Pancreatic Cancers Revealed by Global Genomic Analyses. Science (80-). 2008; 321: 1801–1806. doi: 10.1126/science.1164368
18. Borisenkov MF, Bazhenov SM. Survival in Human Breast Cancer: Effects of Tumor Laterality and the Time of Year of Surgery. 2001; 27: 631–634.
19. Hartveit F, Tangen M, Hartveit E. Side and Survival in Breast Cancer. Oncology. 1984; 41: 149–154. doi: 10.1159/000225812 PMID: 6728398

20. Cappello F, Bellafiore M, Palma A, Marciano V, Zummo G, Farina F, et al. Study of axillary lymph node asymmetry in a female population. J Anat. 2001; 199: 617–620. doi: 10.1046/j.1469-7580.2001.19950617.x PMID: 11760893

21. Dane S, Yildirim S, Koc M, Aktan M, Gundogdu C, et al. Asymmetries in Breast Cancer Lateralization and Both Axillary Lymph Node Number and Metastatic Involvement. Lymphology. 2008; 41: 75–79. PMID: 18720914

22. Veltmaat JM, Ramsdell AF, Sterneck E. Positional variations in mammary gland development and cancer. Journal of Mammary Gland Biology and Neoplasia. 2013. pp. 179–188. doi: 10.1007/s10911-013-9287-3 PMID: 23666389

23. Garfinkel L, Craig L. An Appraisal of Left and Right Breast Cancer. 1. 1969;

24. Stone JM, Cruickshank DG, Sandeman TF, Matthews JP. Laterality, maldescent, trauma and other clinical factors in the epidemiology of testis cancer in Victoria, Australia. Br J Cancer. 1991; 64: 132–8. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1977325&tool=pmcentrez&rendertype=abstract PMID: 1677257

25. Delahunt B, Bethwaite P, Nacey JN. Renal cell carcinoma in New Zealand: a national survival study. 1994; 300–309, 1994 Mar.

26. Perkins CI, Hotes J, Kohler BA, Howe HL. Association between breast cancer laterality and tumor location, United States, 1994–1998. Cancer Causes Control. 2004; 15: 637–645. doi: 10.1023/B:CACO.0000036171.44162.5f PMID: 15280621

27. Roychoudhuri R, Putcha V, Meller H. Cancer and Laterality: A Study of The Five Major Paired Organs (UK). Cancer Causes Control. 2006; 17: 655–662. doi: 10.1007/s10552-006-0015-9 PMID: 1663912

28. Hsieh C c., Trichopoulos D. Breast size, handedness and breast cancer risk. Eur J Cancer Clin Oncol. 1991; 27: 131–135. doi: 10.1016/0277-5379(91)90469-T

29. De Blacam C, Ho WL, Acton C, Murphy G, Kneafsey B, Hill ADK. Prevalence of left-sided melanomas in an Irish population. Irish Journal of Medical Science. 2011. pp. 727–730. doi: 10.1007/s11845-011-0713-5 PMID: 21499924

30. Brim H, Kumar K, Nazarian J, Hathout Y, Jafarian A, Lee E, et al. Slc5a8 gene, a transporter of butyrate: A gut flora metabolite, is frequently methylated in african american colon adenomas. PLoS One. 2011; 6: 3–8. doi: 10.1371/journal.pone.0020216

31. Dong SM, Lee EJ, Park CK, Kim K-M. Progressive methylation during the serrated neoplasia pathway of the colorectum. Mod Pathol an Off J United States Can Acad Pathol Inc. 2005; 18: 170–178. doi: 10.1038/modpathol.3800261

32. Fatima N, Zaman MU, Maqbool A, Khan SH, Riaz N. Lower incidence but more aggressive behavior of right sided breast cancer in Pakistani women: Does right deserve more respect? Asian Pacific J Cancer Prev. 2013; 14: 43–45. doi: 10.7314/APJCP.2013.14.1.43

33. Onibokun O, Killelea BK, Chagpar AB, Horowitz NR, Lannin DR. The left sided predominance of breast cancer is decreasing. Breast J. 2015; 21: 213–215. doi: 10.1111/tbj.12385 PMID: 25582054

34. Wilting J, Hagedom M. Left-right asymmetry in embryonic development and breast cancer: common molecular determinants? CurrMedChem. 2011; 18: 5519–5527.

35. Robichaux JP, Hallett RM, Fuseler JW, Hassell JA, Ramsdell AF. Mammary glands exhibit molecular laterality and undergo left-right asymmetric ductal epithelial growth in MMTV-cNeu mice. Oncogene. 34; 2003–10. doi: 10.1038/cncr.2014.149 PMID: 24909172

36. Feil R, Fraga MF. Epigenetics and the environment: emerging patterns and implications. Nat Rev Genet. 2012; 13: 97–109. doi: 10.1038/nrg3142 PMID: 22215131

37. Cortessis VK, Thomas DC, Joan Levine A, Breton CV, Mack TM, Siegmund KD, et al. Environmental epigenetics: Prospects for studying epigenetic mediation of exposure-response relationships. Human Genetics. 2012. pp. 1565–1589. doi: 10.1007/s00439-012-1189-8 PMID: 22740325

38. Waterland RA. Early environmental effects on epigenetic regulation in humans. Epigenetics. 2009. pp. 523–525. doi: 10.4161/epi.4.8.10155 PMID: 19923895