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Loss, mutation and deregulation of *L3MBTL4* in breast cancers

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Abstract

Background: Many alterations are involved in mammary oncogenesis, including amplifications of oncogenes and losses of tumor suppressor genes (TSG). Losses may affect almost all chromosome arms and many TSGs remain to be identified.

Results: We studied 307 primary breast tumors and 47 breast cancer cell lines by high resolution array comparative genomic hybridization (aCGH). We identified a region on 18p11.31 lost in about 20% of the tumors and 40% of the cell lines. The minimal common region of loss (Chr18:6,366,938-6,375,929 bp) targeted the *L3MBTL4* gene. This gene was also targeted by breakage in one tumor and in two cell lines. We studied the exon sequence of *L3MBTL4* in 180 primary tumor samples and 47 cell lines and found six missense and one nonsense heterozygous mutations. Compared with normal breast tissue, *L3MBTL4* mRNA expression was downregulated in 73% of the tumors notably in luminal, ERBB2 and normal-like subtypes. Losses of the 18p11 region were associated with low *L3MBTL4* expression level. Integrated analysis combining genome and gene expression profiles of the same tumors pointed to 14 other potential 18p TSG candidates. Downregulated expression of *ZFP161*, *PPP4R1* and *YES1* was correlated with luminal B molecular subtype. Low *ZFP161* gene expression was associated with adverse clinical outcome.

Conclusion: We have identified *L3MBTL4* as a potential TSG of chromosome arm 18p. The gene is targeted by deletion, breakage and mutations and its mRNA is downregulated in breast tumors. Additional 18p TSG candidates might explain the aggressive phenotype associated with the loss of 18p in breast tumors.

Background

The development and progression of breast cancer is the result of the accumulation of genetic alterations such as amplification of oncogenes and deletions of tumor suppressor genes (TSG) in the epithelial cells of the mammary gland. Frequent deletions have been reported on chromosome arms 1p, 3p, 7q, 8p 9p, 16q and 17p but only few reports describe such deletions on chromosome arm 18p [1-3].

We profiled a series of 307 primary breast tumors and 47 breast cancer cell lines by using high resolution array comparative genomic hybridization (aCGH). We identified a region on 18p11.31 deleted in 25% of the tumors

and 40% of the cell lines. We delineated a minimal common region of deletion that targeted the *L3MBTL4* gene. The *L3MBTL4* gene is one of the four human orthologs of *Drosophila* lethal (3) malignant brain tumor (*l(3)mbt*). *l(3)mbt* is a *bona fide* TSG in the fly [4,5]. The *L3MBTL4* protein contains three "malignant brain tumor" (MBT) domains. This domain of about 100 amino acid residues is conserved in protostomians and deuterostomians and often exists as repeats [6]. The MBT domain binds methylated histone residues. The human genome contains several MBT-containing proteins, some of which have been linked to gene regulatory pathways and polycomb-mediated repression, and to cancer [7]. *L3MBTL1* is a TSG implicated in myeloid malignancies [8], and *L3MBTL3* deregulation is associated with neuroblastoma [9].

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To document the involvement of *L3MBTL4* in breast cancer we searched for mutations by sequence analysis of 180 primary tumor samples and 47 cell lines. We found that, in addition to deletions, *L3MBTL4* is targeted by mutations. Finally, we found that *L3MBTL4* deletions correlate with low mRNA expression and with the presence of lymph node metastasis, high Scarf-Bloom-Richardson (SBR) grade and luminal B molecular subtype.

Our study is the first to identify a region on chromosome arm 18p likely to contain a putative TSG involved in breast oncogenesis, and to show that this TSG may be *L3MBTL4*. We did not exclude the existence of other potential 18p TSGs, some of which could explain the aggressive phenotype of breast tumors with 18p loss.

Samples and Methods

Breast tumors

Tumor tissues were collected from 307 patients with primary adenocarcinoma who underwent initial surgery at the Institut Paoli-Calmettes (Marseille, France) between 1992 and 2004. Immediately after macroscopic examination of the surgery specimen by two pathologists, samples containing more than 60% of tumor cells were obtained, frozen in liquid nitrogen and stored at -80°C until nucleic acids extraction. The main histoclinical characteristics of tumors are listed in the Additional file 1, Table S1. Each patient gave written informed consent and the study was approved by our institutional review committee.

Cell lines

The 47 breast cell lines used in this study were BT-20, BT-483, HCC1937, CAMA-1, HCC38, HCC1500 [10], Hs 578T [11], BT-474, BT-549, HCC202, HCC1395, HCC1569, HCC1806, HCC1954, HCC2218, HME-1, MCF7, MDA-MB-134, MDA-MB-157, MDA-MB-175, MDA-MB-231, MDA-MB-361, MDA-MB-415, MDA-MB-436, MDA-MB-453, MDA-MB-468, SK-BR-3, SK-BR-7, T47 D, UACC-812, UACC-893, ZR-75-1, ZR-75-30 (ATTC, Manassas, VA), BrCa-Mz-01, BrCa-Mz-02 [12], SUM44, SUM52, SUM102, SUM149, SUM159, SUM185, SUM190, SUM206, SUM225, SUM229 [13], S68 (V. Catros, Rennes, France), and CAL51 [14]. All cell lines were grown as recommended by their suppliers.

DNA and RNA extraction

DNA and RNA were extracted from frozen samples by using guanidium isothiocyanate and cesium chloride gradient, as previously described [15]. DNA quality and RNA integrity were respectively controlled on polyacrylamide gel electrophoresis and on Agilent Bioanalyzer (Agilent Technologies, Massy, France). DNA was also

extracted from a normal area of paraffin-embedded T8584, T8847, T9193 and T8525 tissues.

Genome analysis by array-comparative genomic hybridization (aCGH)

Genome profiles were established on 307 tumors and 47 breast cancer cell lines by aCGH using 244K CGH Microarrays (Hu-244A, Agilent Technologies, Massy, France) as previously described [16]. A pool of 13 normal male DNAs was used as reference. Scanning was done with Agilent Autofocus Dynamic Scanner (G2565BA, Agilent Technologies). Data analysis was done and visualized with CGH Analytics 3.4 software (Agilent Technologies). Extraction of data (\log_2 ratio) was done from CGH analytics, while normalized and filtered \log_2 ratio were obtained from "Feature extraction" software (Agilent Technologies). Data generated by probes mapped to X and Y chromosomes were eliminated. The final dataset contained 225,388 unique probes covering 22,509 genes and intergenic regions according to the hg17/NCBI human genome mapping database (build 35). Data were analyzed using circular binary segmentation (CBS) [17] as implemented in the DNA copy R/Bioconductor package [18] with default parameters to translate intensity measurements in regions of equal copy number, each region being defined by at least five consecutive probes. Thus, each probe was assigned a segment value referred to as its "smoothed" value.

We used a threshold value of $|0.33|$ (\log_2 ratio) to define a copy number aberration (CNA) [16]. Identification of copy number variations (CNV) was done using the regions published by McCarroll *et al* [19] which are stored in the Database of Genomic Variants (release v8) [20]. To determine altered regions, we used the GISTIC algorithm [21], which computes for each genomic segment through the whole genome a score based on the frequency of CNA combined with its amplitude, with bootstrapping to calculate the significance level ($p < 0.001$). To establish significant association between CNA and categorical variables, Fisher's exact test was used. Gains and losses were handled separately.

Mutation analysis

Sequence analysis was done on 180 out of the 307 breast tumors and on the 47 breast cell lines after amplification of genomic DNA. Seventeen primers pairs were designed to amplify *L3MBTL4* DNA by polymerase chain reaction (PCR) (Additional file 1, Table S2). PCR amplifications were done in a total volume of 25 μ l PCR mix containing at least 10 ng template DNA, Taq buffer, 500 μ mol of each deoxynucleotide triphosphate, 100 μ mol of each primer and 1 unit of Hot Star Taq DNA polymerase (Qiagen, Courtaboeuf, France). PCR

amplification conditions were as follows: 95°C 10 min and 95°C 30 sec, annealing T° 55°C 30 sec, 72°C 45 sec to 1 min depending on PCR product length, 72°C 10 min, 35 cycles. PCR products were purified using Millipore plate MSNU030 (Millipore SAS, Molsheim, France). Aliquots (1 µl) of the purified PCR products were sequenced using Big Dye terminator v1.1 Cycling Sequencing Kit (Applied Biosystems, Courtaboeuf, France) including the forward or reverse primer. The sequencing products were purified through Multi-Screen-HV 96-well filter plates (Millipore, Billerica, MA, USA) preloaded with Sephadex G-50 (Sigma, St Louis, MO, USA). The reactions were run on the ABI 3130XL Genetic Analyser (Applied Biosystems, Courtaboeuf, France). The sequence data files were analyzed using the phred/phrap/consed software. All mutations were confirmed by a second round of PCR and sequencing reactions in both directions.

Quantitative real time reverse-transcribed PCR (qRT-PCR)

L3MBTL4 gene expression level was analyzed by qRT-PCR on a set of 52 out of the 307 tumors. Two µg of total RNA, treated beforehand with RNase-free DNase (Promega, France), was reverse-transcribed using the SuperScript II RT and 100 ng of randomhexamers (Invitrogen, France). PCR reactions were carried out in a LightCycler 2.0 instrument (Roche, Germany) in a final volume of 20 µl according to the supplier's recommendations using LightCycler FastStart DNA Master^{plus} SYBR Green I Kit (Roche, Germany). *L3MBTL4* primers, *L3MBTL4-F* (CTTGGAGCAAGCTGAAGAGG) and *L3MBTL4-R* (TGGAAAGGACTGATCCTTGG) (Sigma-Aldrich, Austria), were designed to anneal to exon 4 and exon 6, respectively. Primers for the control gene, *GUSB*, were *GUSB-F* (GAAAATATGTGGTTGGAGAGCT) and *GUSB-R* (CCGAGTGAAGATCCCCTTTT) (Sigma-Aldrich, Austria). The mean threshold cycle (Cp) was calculated for each gene and ΔCp was defined as ΔCp = Cp (*GUSB*) - Cp (*L3MBTL4*). The ΔCp was determined on two to six times for each sample and the mean calculated. The fold ratio of *L3MBTL4* transcripts was calculated using the equation, fold ratio = 2^{ΔCp}. Commercial pools of normal breast RNA (Clontech, Palo Alto, CA) were analyzed and used as control.

Gene expression profiling with DNA microarrays

A total of 229 tumors studied by aCGH, as well as 4 normal breast tissue samples, were profiled with Affymetrix U133 Plus 2.0 human oligonucleotide microarrays as previously described [22]. Scanning was done with Affymetrix GeneArray scanner. Data were analyzed by 'Robust Multichip Average' (RMA) with the non-parametric quantile algorithm as normalization parameter in R/Bioconductor and associated packages [18].

All probes were mapped based on their EntrezGeneID. When multiple probes were mapped to the same gene, the probe sets with an extension « at », next « s_at », and followed by all other extensions were preferentially kept. When several probe sets with the best extension were available, the one with the highest median value was retained.

The five molecular subtypes related to the intrinsic breast cancer tumor classification were determined using the single sample predictor (SSP) classifier [23] associated to 'Distance Weighted Discrimination' (DWD) as data set adjustment [24].

Prior statistical analyses *L3MBTL4* expression level in samples was centered using its expression level in the 4 normal breast samples pooled. Over- and under-expression were defined using a two-fold threshold i.e. |1| in the log₂ transformed data.

Comparative analyses of genome and expression data

To identify other potential 18p TSGs we compared the degree of CNA-driven RNA downregulation in 229 of the 307 samples by analyzing the genes common to the genome and expression platforms (aCGH Agilent Technologies and Affymetrix) and retained after filtering based on the expression level. Briefly, a potential TSG had to show a lower expression value in a sample with loss than in those without (Student t test) and an under-expression frequency overrepresented in samples with the loss (Fisher's exact test). For both tests significance level was 5% with a false discovery rate (FDR, [25]) less than 1%.

Statistical analyses

Correlations between *L3MBTL4* CNA groups and histoclinical factors were calculated with the Fisher's exact test. Student's *t* test and one-way ANOVA test were used to evaluate association of *L3MBTL4* gene expression level within histoclinical factors. Overall specific survival (OS) and metastasis-free survival (MFS) curves were estimated using the Kaplan-Meier method and statistical significance of pairwise comparisons was assessed using the log-rank test. OS and MFS follow-up times were measured from the date of diagnosis till death from breast cancer and till the first occurrence of distant metastases, respectively. All statistical tests were two-sided at the 5% level of significance. Statistical analysis was done using the survival package (version 2.30), in the R software (version 2.9.1).

Results

Losses and breakages of 18p targets the *L3MBTL4* gene at p11.31

Genome profiles of 307 primary breast tumors and 47 cancer cell lines were established by aCGH. GISTIC

analysis showed that chromosome arm 18p was targeted by losses in more than 20% of tumor samples (Additional file 2, Figure S1). None of these losses were homozygous deletions. Profiles showed various sizes of losses (Figure 1A). In tumor T8700 loss within 18p11.31 (Figure 1A) spanned the *L3MBTL4* gene only (Figure 1B). 18p losses including this region were found in 77 (25%) primary tumors and 19 (40%) cell lines.

Copy number transitions are likely to reflect DNA strand breakages that may lead to nonreciprocal translocations [26]. *L3MBTL4* was targeted by copy number transition in tumor T50115, and in the HCC38 (Figure 1C, D) and MDA-MB-453 cell lines. In T50115, the breakpoint was within the genome interval [chr18: 6,202,799-6,212,615], which contains *L3MBTL4* exons 10 and 11. The resulting protein should be truncated of its C-terminal part starting from the second MBT motif. In HCC38 and MDA-MB-453 the breakpoints spanned

genome intervals chr18: 6,009,803-6,020,061, (between exons 17 and 18) and chr18: 6,030,519-6,044,093 (including exon 16), respectively. These two breakages should generate a protein truncated of the sterile alpha motif (SAM) domain. None of these potential fusion transcripts involving the *L3MBTL4* gene has been identified yet.

Thus, the *L3MBTL4* gene is targeted by various genomic alterations, including loss and breakage, in a high proportion of breast cancers.

Features of tumors with *L3MBTL4* loss

L3MBTL1, *L3MBTL2* and *L3MBTL3* paralogs were not targeted by deletion in our breast tumor set. We did not find any *L3MBTL4* deletion among genome profiles similarly established in 80 colon cancers, 115 myeloid hematopoietic diseases and 53 sarcomas (data not shown). This suggests that *L3MBTL4* loss occurs specifically in breast cancers.

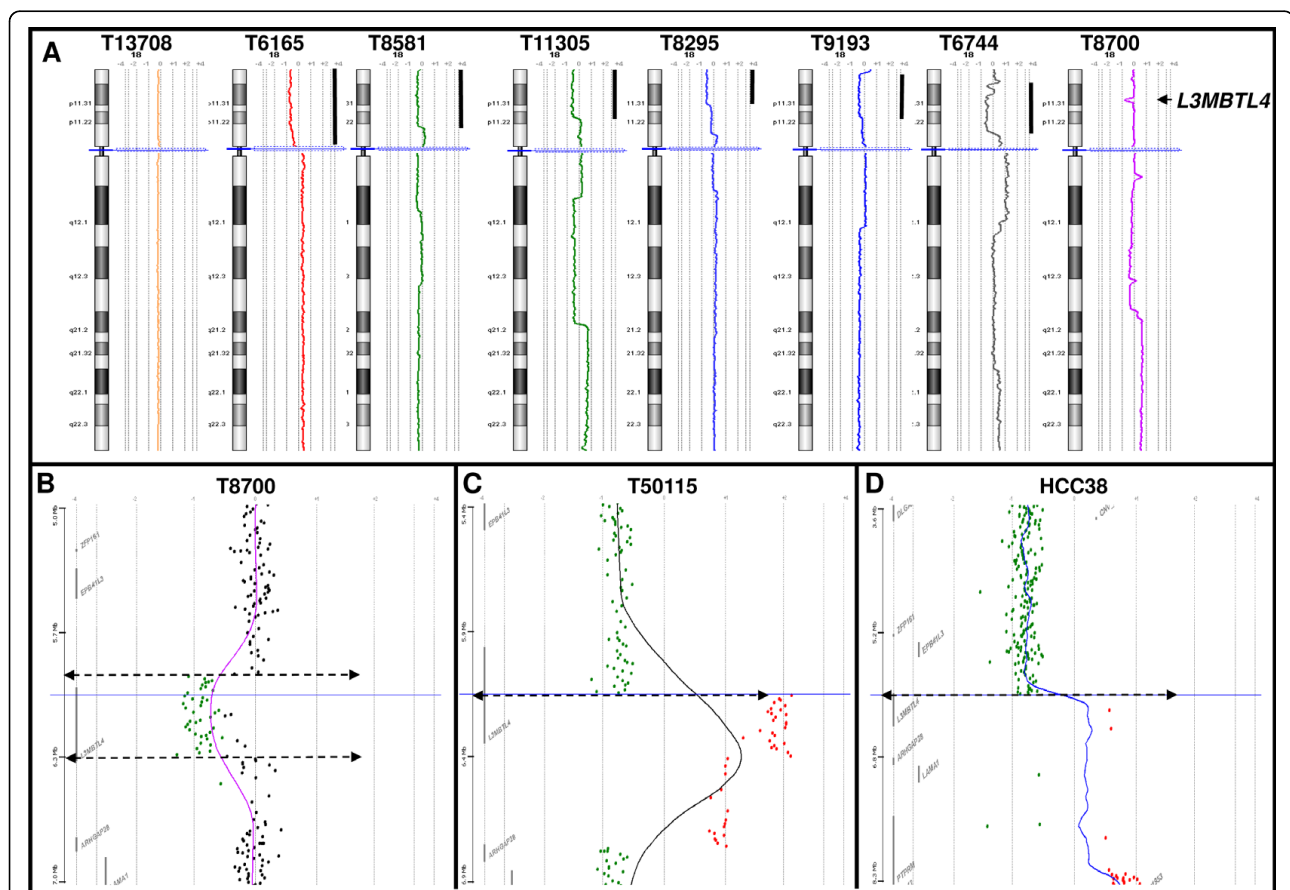


Figure 1 Examples of chromosome 18 aCGH profiles. (A) Tumor T13708 on the left does not present any gene copy number aberration. Tumors T6165, T8581, T11305, T8295, T9193, T6744 and T8700 exhibit copy number losses on the short arm of chromosome 18. The genomic profile observed in tumor T8700 shows the smallest region targeted by the 18p loss. (B) The smallest deletion observed in breast primary tumor T8700 targets the *L3MBTL4* gene (18p11.31) and includes the minimal 18p common lost region, Chr18:6,366,938-6,375,929 bp defined with 96% of studied tumors (Additional file 1, Table S4A). (C, D) Breakpoint targeting the *L3MBTL4* gene in breast primary tumor T50115 and HCC38 breast cancer cell line.

Comparison of aCGH data in tumors with and without *L3MBTL4* loss showed that *L3MBTL4* loss was often found together with the loss of four 17q genes: *ACCN1* ($p = 2.5 \cdot 10^{-5}$), *RHOT1* ($p = 8.3 \cdot 10^{-5}$), *PSMD11* ($p = 1.3 \cdot 10^{-4}$), and *MYO1D* ($p = 1.8 \cdot 10^{-4}$) (Additional file 1, Table S3).

The 77 tumors with *L3MBTL4* loss comprised 18 luminal A, 27 luminal B, 17 basal, 9 ERBB2, 2 normal-like (Table 1) and 4 non-informed tumors (Additional file 1, Table S4A). Among these, 42 (55%) showed loss of a regional part of chromosome 18p including the *L3MBTL4* gene. They included 8 luminal A, 15 luminal B, 10 basal, 5 ERBB2, 1 normal-like and 3 non-informed tumors. The minimal commonly deleted region (CDR) including *L3MBTL4* was defined for each molecular subtype as chr18:6,366,938-6,798,785 bp in luminal A (telomeric and centromeric limits were defined by tumors T11348 and T8683, respectively and noted [T11348-T8683]), chr18:3,784,366-6,202,799 bp in luminal B ([T9377-T50115]), chr18:6,285,396-6,375,929 bp in basal ([T5142cc-T8700]), chr18:3,732,722-6,168,500 bp in ERBB2 ([T9296]), chr18:5,520,302-8,628,361 bp in normal-like ([T12652]) and chr18:5,200,158-11,854,021 bp in non-informed tumors ([T6744-T12662]) (Additional file 1, Table S4A).

The 35 other cases showed a loss of *L3MBTL4* as a consequence of the complete loss of the chromosome 18p. They included 10 luminal A, 12 luminal B, 7 basal, 4 ERBB2, 1 normal-like and 1 non-informed tumors. Interestingly, the complete loss of the chromosome 18p was associated with the luminal B molecular subtype ($p < 0.05$), as well as cancer in older women (>50 years old) (Additional file 1, Table S4B).

Taken together, a minimal 18p CDR, chr18:6,366,938-6,375,929 bp within the *L3MBTL4* locus, was defined in 74 tumors (Additional file 1, Table S4A). In T9296, T50115 and T50136 the *L3MBTL4* loss flanked this CDR at the telomeric and centromeric borders, respectively.

Comparison of clinical features between breast tumors with and without *L3MBTL4* loss (Table 1) showed that *L3MBTL4* loss was associated with the presence of lymph node metastases ($p = 1.02 \cdot 10^{-2}$), high SBR grade ($p = 1.15 \cdot 10^{-2}$) and luminal B molecular subtype defined by SSP classification [23] ($p = 3 \cdot 10^{-5}$). No impact on survival was noted.

L3MBTL4 is targeted by mutations

We searched for mutation in *L3MBTL4* exons in 180 and 47 of the aCGH-profiled tumors and cell lines, respectively. Sequence analysis of the tumor samples identified 32 variants including 25 synonymous (23 p.Ileu570Ileu, 2 p.Val201Val), 6 missense (3 p.Ser123Asn, 1 p.Ser493Leu, 1 p.Glu560Lys, 1 p.Ile615Ser) and 1 nonsense (p.Tyr339X). In BT-483 cell line a missense

(p.Arg96Gln) mutation was identified. Figure 2A shows two examples of mutation. The localization and nature of the mutations are shown in Figure 2B. The p.Ser123-Asn substitution and the nonsense mutation were located in regions encoding the conserved MBT1 and MBT3 motifs, respectively. The nonsense mutation should generate a truncated *L3MBTL4* protein without C2HC zinc finger and SAM domains. The p.Arg96Gln substitution in BT-483 should affect the MBT1 domain. The SAM motif was also targeted by the p.Glu560Lys substitution. We were able to show that the p.Ser123-Asn and p.Tyr339X mutations found respectively in T8584, T8847 and T9193 and in T8525, were acquired (Figure 2A). We could not confirm that the other missense mutations are similarly somatic. However, they were not included as SNP or missenses in NCBI db SNP build 131 [27].

Counting only the 7 potentially deleterious mutations the frequency of such event would be 3.9% in tumor samples.

Two mutated (missense mutation) tumors (T9193 and T10003) were also deleted.

L3MBTL4 mRNA is downregulated in breast tumors

L3MBTL4 gene expression was measured using qRT-PCR in normal breast tissues and 52 breast tumor samples including 16 with a loss of the *L3MBTL4* gene region (T7420, T8009, T8189, T8600, T8700, T9059, T9398, T9888, T9941, T11348, T11485, T11568, T10684, T12854, T13469, T13018), one mutated and deleted (T10003), and one mutated but not deleted (T8525). *L3MBTL4* gene expression was quantified by comparison with the expression of the housekeeping *GUS* gene. Overall, tumor samples expressed a low level of *L3MBTL4* mRNA as compared with normal breast tissue. *L3MBTL4* mRNA level was decreased at least two-fold in all tumors with *L3MBTL4* loss as well as in the mutated and non-deleted sample (Figure 3). Some non-deleted tumors also exhibited low *L3MBTL4* mRNA level suggesting that *L3MBTL4* downregulation could be attributed to several mechanisms including deletions but also regulation of transcription.

We also determined *L3MBTL4* gene expression in 229 tumor samples and 4 NB samples by using Affymetrix microarrays. *L3MBTL4* mRNA was downregulated (ratio T/NB <0.5) in 166 tumors (72%). Comparative analysis of these 229 samples identified a significant correlation ($p = 5.05 \cdot 10^{-4}$) between *L3MBTL4* mRNA downregulation and gene loss (Figure 4A). Downregulation of *L3MBTL4* mRNA was associated with luminal A, luminal B and ERBB2 molecular subtypes ($p = 6.734 \cdot 10^{-43}$).

Deletion, mutation and expression data are described in the Additional file 1, Table S4A and summarized in Table 2.

Table 1 Comparison of clinical features between breast tumors associated or not with *L3MBTL4* loss (determined by aCGH)

Characteristics (N)	<i>L3MBTL4</i> non deleted N = 230	<i>L3MBTL4</i> deleted N = 77	p	odds ratio
Age (300)	52 (22-84)	51 (24-82)	0.69	
Histological type (255)			0.57	
Ductal	152 (78%)	48 (79%)		
Lobular	15 (8%)	5 (8%)		
Medullary	12 (6%)	2 (3%)		
Mixt	8 (4%)	3 (5%)		
Other	7(4%)	3 (5%)		
Clinical form (301)			0.11	1.76 (0.87-3.5)
IBC	33 (15%)	18 (23%)		
Non IBC	191 (85%)	59 (77%)		
Pathological tumor size (240)			0.052	
pT1	45 (25%)	8 (14%)		
pT2	95 (52%)	28 (48%)		
pT3	42 (23%)	22 (38%)		
Pathological axillary lymph node status (266)			1.02E-02	0.46 (0.24-0.85)
Negative	96 (48%)	20 (30%)		
Positive	103 (52%)	47 (70%)		
SBR grade (267)			1.15E-02	
1	38 (19%)	3 (5%)		
2	63 (31%)	25 (38%)		
3	100 (50%)	38 (58%)		
ER (275)			0.11	0.6 (0.31-1.13)
Negative	84 (40%)	19 (29%)		
Positive	125 (60%)	47 (71%)		
PR (264)			0.32	0.74 (0.4-1.36)
Negative	96 (48%)	26 (41%)		
Positive	104 (52%)	38 (59%)		
P53 (192)			0.39	1.38 (0.65-3)
Negative	87 (59%)	30 (67%)		
Positive	60 (41%)	15 (33%)		
Ki67 (217)			0.23	0.64 (0.28-1.36)
Negative	53 (32%)	12 (23%)		
Positive	112 (68%)	40 (77%)		
ERBB2 (226)			1	1.03 (0.42-2.83)
Negative	145 (85%)	48 (86%)		
Positive	25 (15%)	8 (14%)		
Molecular subtype (264)			8.04E-04	
Basal	63 (33%)	17 (23%)		
ERBB2	22 (12%)	9 (12%)		
Luminal A	52 (27%)	18 (25%)		
Luminal B	29 (15%)	27 (37%)		
Normal-like	25 (13%)	2 (3%)		
Metastatic relapse (198)			0.32	0.62 (0.23-1.78)
no	135 (88%)	37 (82%)		
yes	18 (12%)	8 (18%)		

Table 1 Comparison of clinical features between breast tumors associated or not with *L3MBTL4* loss (determined by aCGH) (Continued)

Death from breast cancer (239)			0.38	0.73
no	139 (77%)	41 (71%)		(0.36-1.52)
yes	42 (23%)	17 (29%)		
5 year-Metastasis-free survival (198)	90%	81%	0.254	
5 year-Overall specific survival (239)	78%	73%	0.271	

Note: IBC, Inflammatory Breast Cancer; SBR, Scarf-Bloom-Richardson, histological grade.

Expression microarray profile revealed five genes (*TTMA*, *PTPN2*, *NDC80*, *SLMO1* and *TUBB6*) coordinately deregulated with *L3MBTL4* and also localized in an 18p region frequently lost in breast tumors, suggesting a common mechanism of deregulation of their expression, which might contribute to breast oncogenesis.

Correlations between *L3MBTL4* microarray data and clinical features of 229 tumors are reported in the Additional file 1, Table S5. The lowest expression of *L3MBTL4* was found in lobular tumors ($p = 7,11.10^{-12}$) and in inflammatory breast cancers ($p = 0.04$) and was associated with the expression of estrogen receptor (ER) ($p = 2,73.10^{-14}$), progesterone receptor (PR) ($p = 3,11.10^{-13}$), ERBB2 ($p = 7,73.10^{-14}$), SBR grade 2 ($p = 1.49 \cdot 10^4$), lymph node metastasis ($p = 3,39.10^{-5}$), P53 negativity ($p = 0.0495$) and low expression of Ki67 proliferative index ($p = 19,61 \cdot 10^{-5}$).

Sixty-four genes are targeted by 18p copy number losses in breast cancer

To identify other 18p genes targeted by copy number losses, a GISTIC analysis was done on the genome profiles of the 307 cases. Recurrent copy number losses targeted 64 genes within the 18p11.21-11.32 region ($p < 1 \times 10^{-5}$) (Additional file 2, Figure S1), including *L3MBTL4* and the potential TSG *EPB41L3/DAL1* [28]. This suggests that this 18p11 region probably hosts multiple TSGs.

Fourteen new 18p TSG candidates

To identify these other potential 18p11 TSGs we compared the degree of CNA-driven RNA downregulation in the 307 samples by analyzing the genes common to the genome and expression platforms (aCGH Agilent Technologies and Affymetrix) and retained after filtering based on the expression level. Sixty-three of the 64 were common. The gene expression of 14 genes (*YES1*, *SMCHD1*, *LPIN2*, *MRCL3*, *MRCL2*, *ZFP161*, *RALBP1*, *PPP4R1*, *NAPG*, *AFG3L2*, *SPIRE1*, *CEP76*, *PTPN2* and *SEH1L*) was downregulated in relation with their copy number losses ($p < 0.05$) (Additional file 1, Table S6). We noted that *EPB41L3/DAL1* and *L3MBTL4* were not retained. The lowest expression of *EPB41L3* in breast

tumors was associated with the presence of lymph node metastases ($p < 0.05$), correlated with basal and luminal B molecular subtypes ($p < 0.01$) and showed clinical impact (Additional file 1, Table S7).

Among the 14 genes, the downregulated expressions of *ZFP161*, *PPP4R1* and *YES1* in breast tumors were correlated with the luminal B molecular subtype ($p < 0.01$). Interestingly, the downregulated expression of *ZFP161* was correlated with a poor clinical outcome (5-years MFS, $p = 1.7.10^{-3}$; 5-years OS, $p = 1.6.10^{-3}$) (Additional file 1, Table S8).

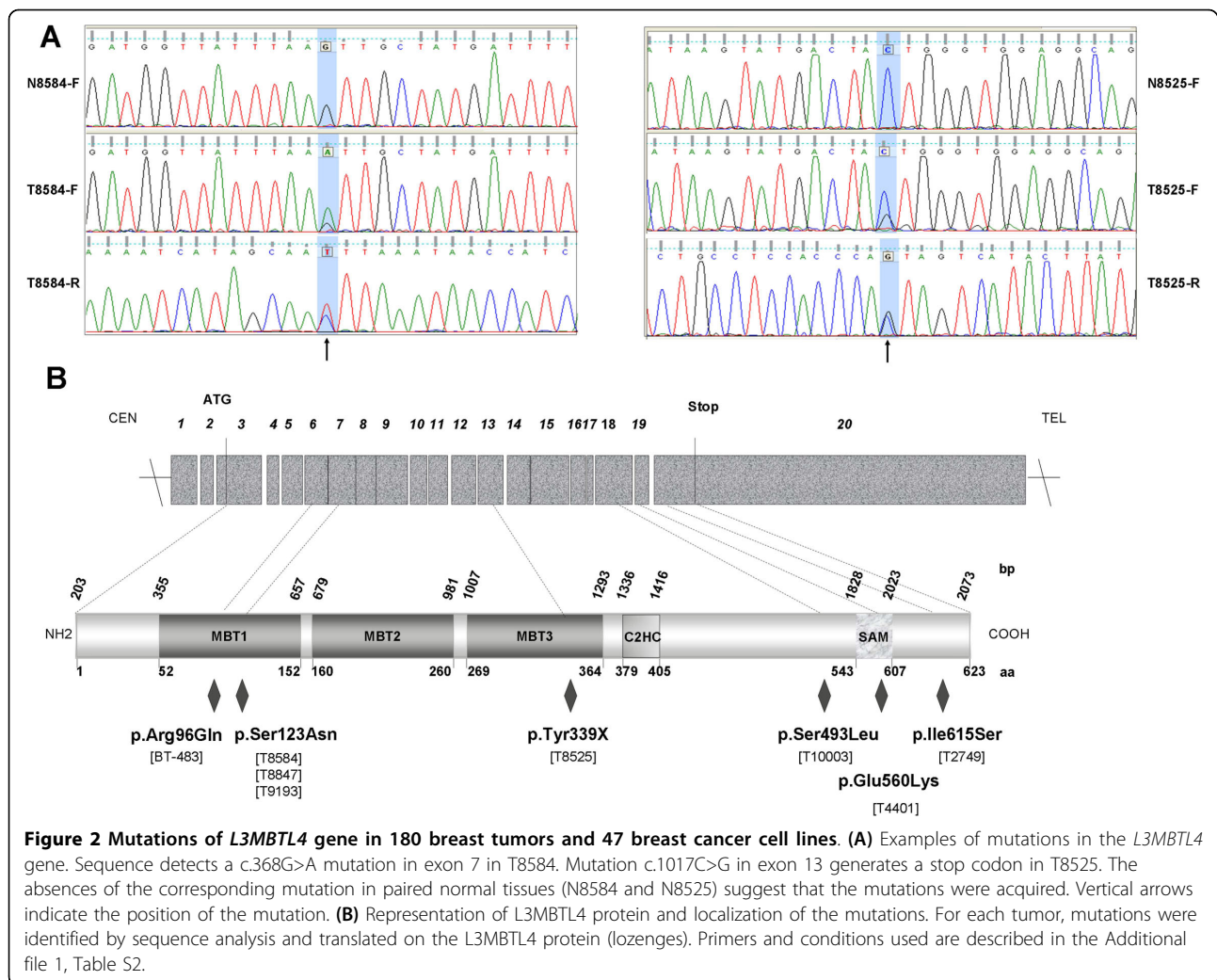
Discussion

L3MBTL4 gene alterations

Few works have reported the presence of TSGs on 18p for breast cancer [1,2] and only *EPB41L3/DAL1* [28] has been identified as potential TSG involved in this neoplasm thus far. Our aCGH results provide a detailed view of the alterations of chromosome arm 18p in breast tumors. Losses at 18p11.31 occurred in 25% of tumors and the smallest common region of deletion targeted the *L3MBTL4* locus and was also the site of breakages. The karyotype of the MDA-MB-453 cell line displays a der(18)t(7;18) [29]. The *L3MBTL4* breakpoint suggests involvement of this gene in this alteration. *L3MBTL4* loss was associated with high grade and with lymph node metastasis. In agreement, an increased risk of relapse in patients with high risk breast cancer has been associated with 18p loss [30]. *L3MBTL4* loss was associated with the luminal B subtype, which is characterized by a poor prognosis. Interestingly, *L3MBTL4* is centromeric and in close proximity to the loss of heterozygosity (LOH) region spanning *EPB41L3* previously reported in non-small cell lung carcinomas [1], breast carcinomas [3], and meningiomas [31].

L3MBTL4 was targeted by point mutations in few cases. These mutations clustered in the vicinity of the MBT or SAM motifs. A study of *L3MBTL1* has shown that a point mutation in the second MBT repeat motif affected the binding to H1K26me and H4K20me [32]. We surmise that the nonsense mutation in the third MBT motif of *L3MBTL4* may have similar functional implication.

In three tumors and in the BT-483 cell line *L3MBTL4* mutations were associated with the loss of the other



allele, suggesting a double-hit mechanism leading to complete loss-of-function of *L3MBTL4* in these samples. We did not find homozygous mutations or deletions of *L3MBTL4*. However, we cannot exclude that in addition to these alterations, total inactivation could result from promoter hypermethylation of the other allele. The study of mRNA expression tended to confirm this.

L3MBTL4 loss of expression

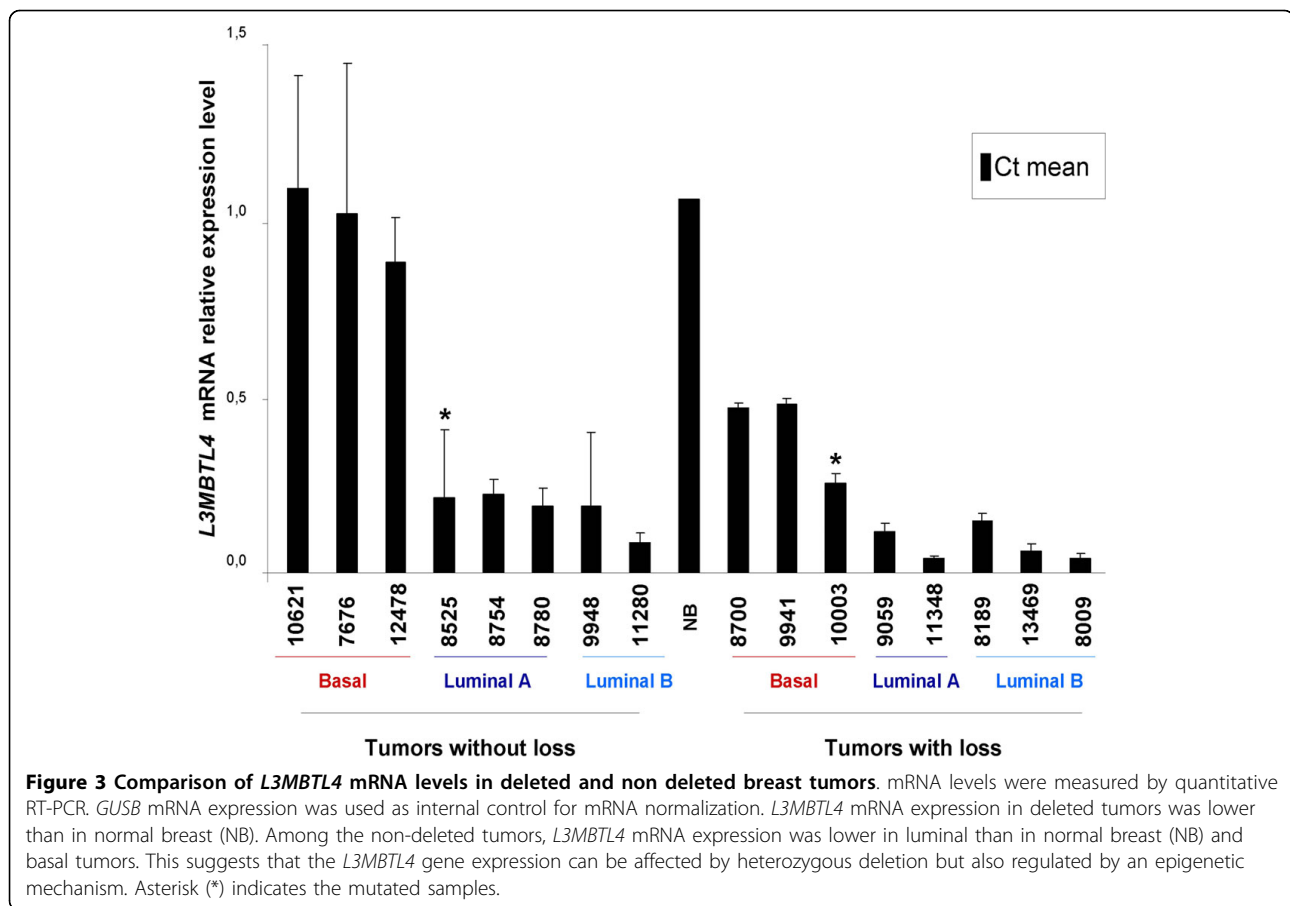
Expression of *L3MBTL4* level was decreased at least two-fold in 73% of breast cancer samples, particularly in tumors deleted at the *L3MBTL4* locus since decreased expression correlated with genomic alteration. All the deleted tumors showed absence or decreased *L3MBTL4* expression. Few cases displayed mRNA downregulation in the absence of loss suggesting that other mechanisms such as epigenetic repression play a role in the downregulation of *L3MBTL4* in breast tumors.

L3MBTL4 downregulated expression was not found only in luminal B tumors but also in luminal A, luminal

B, ERBB2 and normal-like cases. Only the basal subtype presented a normal expression of the gene, independently of loss. This suggests that *L3MBTL4* does not play a TSG role in this subtype of breast cancer. Inactivation of other TSGs may explain 18p losses in basal breast cancers.

L3MBTL4 as a tumor suppressor gene

Our observations are in agreement with a previous study that suggested the presence of TSGs on 18p with a role in the genesis of breast cancer [33]. There are several reasons to believe that *L3MBTL4* is a good candidate TSG. First, it lies within the region of chromosome 18 that is frequently deleted in breast cancers; *L3MBTL4* is also targeted by mutations and breakages and is downregulated in tumors. Second, *L3MBTL4* gene is a human homolog of *Drosophila l(3)mbt*, which functions as a TSG in fly. The loss-of-function generated by mutation of *l(3)mbt* causes brain tumors in *Drosophila* [4,5]. Third, *L3MBTL4* has three paralogs that are suspected



to play a role in the etiology of certain types of cancer [7]. *L3MBTL1*, a known transcriptional repressor [34], has been proposed as a TSG gene in myeloid malignancies associated with 20q deletion [8,35,36]. A recent study reported focal hemi- and homozygous deletions of *L3MBTL2* and *L3MBTL3* in medulloblastoma [9].

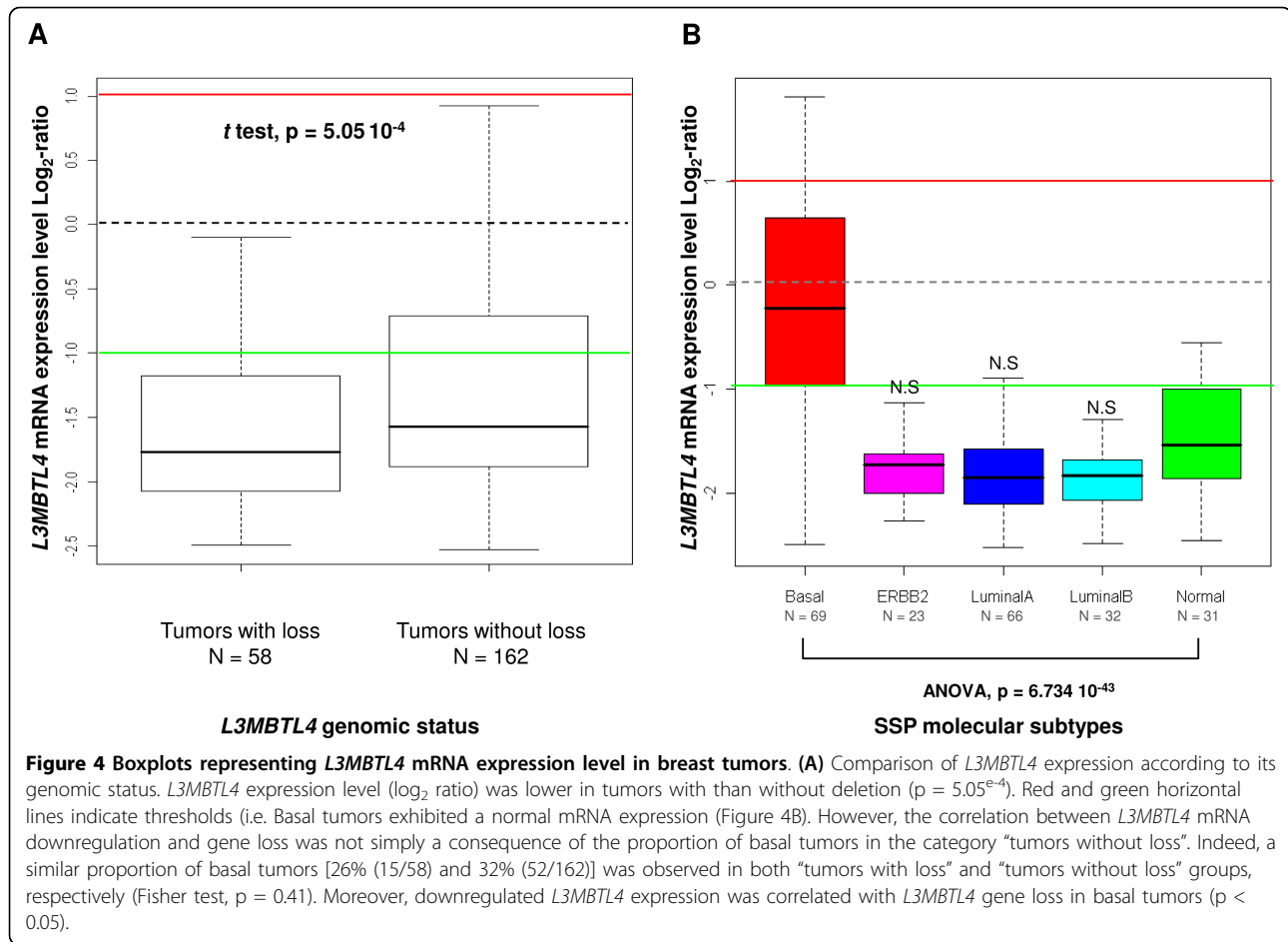
The four human L3MBTL proteins have MBT domains involved in transcriptional repression and chromatin remodeling. The MBT domain was originally identified in the *Drosophila* l(3)mbt protein [5] and binds methyl-lysine residues [37,38], particularly and strongly H3K9me and H4K20me [39]. The transcriptional repressor L3MBTL1 requires its three MBT domains for compacting chromatin and silencing [32]. Although the exact biochemical properties and cellular functions of L3MBTL4 are unclear to date, the presence of MBT domains in L3MBTL4 suggests that it interacts with chromatin [40], may potentially bind methylated histone and thus play a role in transcriptional regulation of stem cell genes, oncogenes and tumor suppressors. Loss of this regulator may thus affect several breast cancer genes. Some of these genes may be part of the E2F/RB pathway. Indeed, the E2F/RB pathway is altered in

luminal B cancers and L3MBTL proteins are known to regulate this pathway [7].

Taken together, our results suggest that aberrations targeting *L3MBTL4* could confer to cancer cells specific advantages but do not exclude the role of other potential 18p candidates.

Other 18p TSG candidates

One of the potential 18p TSGs might be *EPB41L3/DAL1* [28], which is just telomeric to *L3MBTL4*, between the D18S59 and D18S452 markers. However, its expression was not correlated with that of *L3MBTL4*. We also identified 14 TSG candidates whose gene expression was downregulated in relation with their copy number losses. We noted that *EPB41L3/DAL1* and *L3MBTL4* were not included in the 14 suggesting that their downregulated gene expression is not only the consequence of their copy number loss but could result from other mechanisms of deregulation including epigenetic modifications. This is in agreement with study showing that the hypermethylation of *EPB41L3/DAL1* was associated with its downregulation in lung cancer [41]. Our *EPB41L3/DAL1* expression data showed that



its downregulation could be associated with an increased risk of relapse in patients with high risk breast cancer.

EPB41L3/DAL1 undergoes allelic losses in various cancers and in a significant proportion of ductal carcinomas *in situ* of the breast. The EPB41L3/DAL1 protein suppresses the growth of MCF7 breast cancer cells and increases attachment of these cells to a variety of

extracellular matrices [28]. Modulation of post-translational methylation may be an important mechanism through which EPB41L3/DAL1 affects tumor cell growth [42]. EPB41L3/DAL1 plays a critical role in the suppression of lung tumor formation and metastasis [43]. However, the role of *EPB41L3/DAL1* as a TSG has yet to be validated *in vivo*. EPB41L3/DAL1 deficient

Table 2 Summary of *L3MBTL4* alterations

Samples	Loss	Mutation	Loss and mutation	Breakages	*mRNA downregulation
Total tumors (N = 307; *229)	77	7/(180 studied)	2	1	166
Luminal A (N = 70; *66)	18	1	0		62
Luminal B (N = 56; *47)	27	1	0	1(T50115)	45
ERBB2 (N = 31; *23)	9	2	1		22
Basal (N = 80; *67)	17	1	1		16
Normal-like (N = 27; *26)	2	0	0		21
Not informed (N = 43)	4	2	0		0
Total cell lines (N = 47)	19	1	1	2	Not applicable
Luminal cell lines(N = 17)	12	1	1 (BT-483)	1 (MDA-MB-453)	Not applicable
Basal cell lines (N = 9)	2	0	0	1 (HCC38)	Not applicable
Others/Not done (N = 21)	5	0	0	0	Not applicable

* Samples analyzed by using DNA microarrays.

mice are healthy and do not develop spontaneous tumors [44]. Mutational screening failed to identify inactivating mutation of the *EPB41L3/DAL1* gene [33].

Among the 14 other TSG candidates, except *PTPN2/TCPTP* none has been so far associated with cancer. *PTPN2/TCPTP* codes for the T-cell protein tyrosine phosphatase (TCPTP) and is an important negative regulator of SFK, JAK1 and STAT3 signaling during the cell cycle [45]. TCPTP suppresses the tumorigenicity of glioblastoma cells expressing a mutant epidermal growth factor receptor [46]. *CEP76* encodes a centrosomal protein controlling centrosome duplication during cell division. Abnormal centrosome duplication contributes to mitotic failure, genome instability, aneuploidy, and cancer. Depletion of *CEP76* drives the accumulation of centrosome intermediates in certain types of cancer cells [47]. Only the downregulated expressions of *ZFP161*, *PPP4R1* and *YES1* were correlated with the luminal B subtype suggesting their potential involvement in the genesis of a particularly aggressive form of breast cancer with 18p loss. The downregulated expression of *ZFP161* in breast tumors was correlated with a poor clinical evolution. *ZFP161/ZF5* encodes a ubiquitously-expressed protein originally identified by its ability to bind and repress the murine *Myc* promoter [48,49]. The protein contains an N-terminal POZ domain, which recruits cofactors to modulate transcription [50]. *ZFP161/ZF5* mediates both transcriptional activation and repression of cellular and viral promoters [48,50,51]. *ZFP161* may compete with MYC-induced transcription [52].

PPP4R1 encodes the regulatory subunit of a ~125-kDa protein phosphatase. *PPP4R1* interacts with *PPP4C* [53,54] which is implicated in the regulation of histone acetylation, DNA damage checkpoint signaling, NF κ B activation, and microtubule organization at centrosomes [55-59]. *YES1* encodes a SRC-family kinase [60] and its tyrosine kinase activity has been shown to be elevated in colonic adenomas compared to its activity in adjacent normal mucosa [61]. A number of studies have linked increased expression of YES in cancer with increased cell motility and tumor invasion [62,63]. It is then surprising to find *YES1* among the 18p TSG candidates. The down-regulation of *YES1* could be the consequence of its loss as a simple passenger of a larger region lost within 18p.

Conclusion

We have delineated a region of frequent loss in breast cancer on chromosome arm 18p. We have identified *L3MBTL4* as the gene targeted by these losses. *L3MBTL4* is also targeted by mutations and breakages. *L3MBTL4* mRNA expression is low in non-basal breast tumors and in particular in tumors with loss of the gene. Alteration of *L3MBTL4*, coding for a regulator of epigenetic marks, is well in line with recent advances in

cancer research [64,65]. We have also pointed to other 18p TSG candidates including *ZFP161*, *CEP76*, *PPP4R1* and *PTPN2* whose involvement might explain the aggressive phenotype of breast tumors with 18p loss.

Additional material

Additional file 1: Supplementary Tables S1-S8. Table S1. Clinical and histological features of 307 breast tumor cases. Table S2. Primers used for *L3MBTL4* sequence determination. Table S3. Association of genes with copy number alterations and *L3MBTL4* genomic loss. Table S4A. The 77 tumors with *L3MBTL4* loss and mutation. Table S4B. Correlations between clinical features of 305 BC and the loss of whole chromosome 18p. Table S5. Correlations between clinical features of 229 BC and *L3MBTL4* gene expression. Table S6 - Other potential 18p TSG candidates. Table S7. Correlations between clinical features of 260 BC and *EPB41L3* gene expression. Table S8. Correlations between clinical features of 260 BC and down regulated *ZFP161* gene expression.

Additional file 2: Supplementary figure. Figure S1. The 18p is targeted by losses in breast cancer. On the top, combining the CNA frequency and gene copy number alterations level, the GISTIC algorithm plotted the score index observed in genomic profiles of 307 breast tumors as a function of chromosome 18 locations. The chromosome arm 18p was targeted by losses in more than 20% of tumor samples. The dotted line indicates the threshold of significance for the score. At the bottom, the figure shows only an 18p11.21-p11.32 region of 12.79 Mb including 64 loci as significant 18p losses ($p < 10^{-5}$). *L3MBTL4* and *EPB41L3* are contained within this region.

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Authors' contributions

LAK and NC did sequence and qRT-PCR analyses, JA, IB, FS and AF aCGH experiments and analysis, PF and FB gene expression analysis, PV and SM supervised the study, DB and MC designed and supervised the study, LAK and MC wrote the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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