Journal of Carcinogenesis & Mutagenesis

Euhus et al., J Carcinog Mutagen 2018, 9:3
DOI: 10.4172/2157-2518.1000322

Research Article Open Access

Molecular Evolution of Spontaneously Immortalizing Human Mammary Epithelial Cells from a Woman with a Germline STK11 Mutation

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Received date: September 23, 2018; **Accepted date:** October 15, 2018; **Published date:** October 19, 2018

Abstract

Background: Breast carcinogenesis is a multistep process, but the specific molecular steps are not well defined. The canonical view, from immortalization in culture, is that cells escape an initial proliferation blockade by methylating CDKN2A and then eventually acquire extensive DNA changes due to telomere shortening.

Aim: To develop a comprehensive view of the molecular changes accompanying the transition from normal to immortal breast epithelium.

Materials and methods: A comprehensive "OMICS" approach was used to study human mammary epithelial cells from initial culture to spontaneous immortalization. The immortalizing cells were from a woman with Peutz-Jeghers Syndrome (HMEC PJ4719). Controls included an hTERT-immortalized HMEC and early passage HMEC from a fibro-adenoma patient. OMICS platforms included whole genome expression, methylation array, miRNA array, whole exome sequencing and comparative genomic hybridization.

Results: HMEC PJ4719 carried an STK11 c.256C<T p.R86X mutation, but the transcriptomes and methylomes of early passage cells were nearly indistinguishable from control cells. Principle component analysis identified gene expression changes characterizing each phase of immortalization. Gene Set Enrichment Analysis showed homology (P<10-20) with several breast cancer-associated gene sets confirming clinical relevance of the *in vitro* observations. The most profound change during immortalization was global hypomethylation of non-CpG island DNA and regional hypermethylation of CpG islands. Immortalization was associated with a modest accumulation of mutations and DNA copy number alterations but no evidence of general genomic instability. Interestingly, mid-passage cells exhibited DNA deletions that were not seen in the immortal cells, suggesting that mid-passage bulk cell populations were not direct precursors of the immortal clone. Immortalized cells were incapable of anchorage-independent growth or growth in nude mice.

Conclusions: Immortalization may result from adaptive changes evoked by growth in a "stressed" environment. Epigenetic reprogramming rather than mutation is a central feature that may be exploitable for tissue-based breast cancer risk stratification and management.

Keywords: Breast neoplasms; Human mammary epithelial cells; Cell immortalization; DNA methylation; Mutations; DNA copy number

Introduction

The process by which normal breast epithelium transforms into breast cancer is poorly understood. The common conception is that accumulation of random mutations confers a survival advantage that leads to clonal expansion of populations with the capacity to commandeer the micro-environment and disseminate. The frequent association of high risk preneoplasia and in situ carcinoma with fully developed invasive cancer suggests that carcinogenesis can be a protracted process involving diverse cell populations. Normal tissue in the vicinity of breast cancer shows evidence of clonal epigenetic reprogramming towards reduced differentiation [1], while atypical hyperplasia and ductal carcinoma in situ show many of the same genetic [2] and epigenetic [3] changes that characterize invasive breast cancer. We used a comprehensive "OMICS" approach to observe the sequential molecular changes associated with spontaneous immortalization of benign human mammary epithelial cells (HMEC) in culture.

Human mammary epithelial cells cultured in serum-free media will divide only a few dozen times before experiencing a first growth arrest termed M0 or selection. The canonical view is that this first stasis barrier is retinoblastoma-mediated as silencing of CDKN2A (usually by promoter region methylation) permits escape from M0 which is followed by a period of rapid cell division known as post-selection. The canonical view is that rapidly dividing post-selection cells begin to accumulate a large number of genomic defects which ultimately leads to a second period of growth arrest known as M1 or agonescence. This second stasis barrier has been attributed to telomere dysfunction. It is

extremely rare for cells to spontaneously emerge from M1 growth arrest. The cells that do escape M1 are immortal.

We observed spontaneous immortalization of benign HMEC from a patient with Peutz-Jeghers syndrome. This syndrome is caused by germline mutation of STK11. The STK11 protein normally controls the activity of AMP-activated protein kinase (AMPK) family members, ultimately shutting down protein synthesis and cell division when energy stores are insufficient. It also has a role in maintaining cell polarity through remodeling of the actin cytoskeleton. STK11 is recognized as a tumor suppressor gene. Heterozygous carriers of mutated STK11 develop gastrointestinal polyps and are at greatly increased risk for pancreas, gastrointestinal, breast, cervical, uterine, and testicular cancer.

Materials and Methods

Patient materials

This research was performed in compliance with Institutional Review Board regulations governing patient oriented research as well as HIPAA privacy regulations.

A fragment of histologically normal breast tissue was obtained from a 24 year old woman with a clinical diagnosis of Peutz-Jeghers Syndrome (PJS) at the time of partial mastectomy for ductal carcinoma in situ (DCIS). The patient was diagnosed with PJS at the age of three when she underwent small bowel resection for an intussusception caused by hamartomatous polyps. She presented at age 24 requesting breast cancer screening. Her examination at that time was notable only for pigmented freckles on her lips. Her family history was positive for breast cancer in her maternal grandmother at age 65 and fallopian tube cancer in her maternal aunt at age 55 (BRCA1 and BRCA2 tested and negative). Her clinical breast exam was normal but a mammogram showed a 1 cm cluster of micro-calcifications in the left breast that was diagnosed as DCIS on core needle biopsy. Partial mastectomy returned a small focus of DCIS with associated atypical ductal hyperplasia. Sequencing of STK11 in peripheral blood mononuclear cell DNA returned STK11 256C<T R86X which is a pathogenic nonsense mutation in the first exon.

At about the same time, a small fragment of normal breast tissue was also obtained from a 34 year old woman undergoing excision of a fibro adenoma. Normal breast tissue from each woman was minced, enzymatically digested with collagenase I, and cultured in defined serum-free media to select for epithelial cells as previously described [4]. Early passage cells were collected from each of the cultures. A portion of these early passage cells from the PJS patient were immortalized using retroviral human telomerase (hTERT) transduction as previously described [5,6]. Additional samples were harvested from the PJS culture after escape from M0 growth arrest and after spontaneous immortalization.

"OMICS" platforms

Most assays were run on Illumina platforms according to manufacturer protocols. Gene expression was measured using Illumina HT12 V4 Expression arrays; methylation profiling using Infinium® HumanMethylation450 BeadChips; DNA copy number using Illumina HumanOmni1-Quad; and exome sequencing by Agilent Sure Select V4 run on the Illumina Hiseq 2500. miRNA profiling was performed using the Exiqon–miRCURY™ LNA Array.

Statistical methods

Sample preprocessing: Estimates of *mRNA* expression levels were obtained using Illumina Bead Studio software and quantile normalized to correct for unwanted variation between samples before differential expression analysis. The ExiMiR package [7] from Bio-conductor [8] was used to preprocess the Exiqon miRNA array data, using recommended procedures, including normalization using spike-in controls and summary using median polish. Illumina 450K DNA methylation data was preprocessed using the functional normalization algorithm as implemented in the minfi package from Bio-conductor [9] and reported as beta values describing the proportion of methylated DNA at each site. CpG sites within 10 bases of a known SNP were removed from analysis. DNA sequencing reads were aligned to the GRCh37 human reference sequence (http://www.ncbi.nlm.nih.gov/projects/genome/assembly/grc/human/) using Bowtie2 [10]. Variant calling was performed using MuTect [11] with standard parameters, and filtered for a minimum of 20 reads and a mutant allele frequency of at least 0.3. Mutations were annotated using snpEff [12]. Illumina SNP arrays were read into R and segmented using the circular binary segmentation algorithm [13] as implemented in the DNA copy package [14] from Bioconductor followed by analysis using GISTIC2 via the GENEPATTERN site, to infer significant copy number changes. Exploratory data analysis included unsupervised, agglomorative hierarchical clustering using Ward's method, and principle component analysis [15].

Differential expression (*mRNA* and *mi-RNA*) and methylation was calculated using Empirical Bayes linear models as implemented in the limma package from Bio-conductor. Gene set analysis was performed based on curated data sets from MSigDB (http://software.broadinstitute.org/gsea/msigdb/index.jsp) to identify altered pathways, using Wilcoxon tests as implemented in the limma package [15].

Results

Both the PJS and control human mammary epithelial cells (HMEC) initially exhibited a classic growth pattern in culture characterized by several doublings prior to M0 growth arrest, escape from M0 arrest and then several more doublings prior to the M1 growth arrest (Figure 1).

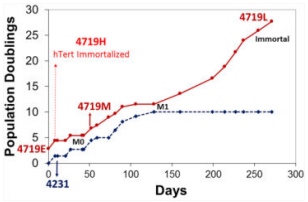

Figure 1: Growth Curves and Sample Time Points. 4719 are Human Mammary Epithelial Cells (HMEC) from a Peutz-Jeghers patient. 4719E are early passage (passage 3) preselection cells; 4719M are from between M0 and M1 growth arrest (post-selection); 4719L are immortalized. 4719H are early passage pre-selection cells that were hTERT immortalized. 4231 are early passage (passage 3) preselection cells.

Unlike the control cells (HMEC 4231), however, the PJS cells (HMEC PJ4719) escaped M1 growth arrest and spontaneously

immortalized. The immortalized cells formed mammospheres in Matrigel culture, but cannot be classified as cancer cells as they were incapable of anchorage-independent growth and did not form tumors in nude mice. They did not express estrogen receptor, progesterone receptor or HER-2/neu.

Gene expression

The gene expression profile of early passage HMEC PJ4719 is nearly identical to that of the control HMEC (Figure 2a). Progression to immortalization is marked by loss of expression of many genes and gain of expression of a few genes (Figure 2 inset). Principle Component Analysis (PCA) identified 4 key components (Figure 2b). The first component shows excellent separation between the samples and the expected order of progression. Gene Set Enrichment Analysis based on the top 130 genes driving the PCA (Supplemental Table 1) identified more than 100 gene sets with adjusted P-values <10-20. The best correlated gene sets were CDH1 targets, targets of SMAD2 or SMAD3, breast cancer basal up, breast cancer basal versus luminal, mammary stem cell up, breast cancer luminal versus mesenchymal, mammary stem cell down, HRAS oncogene signature, ESR1 target down, tamoxifen resistance up, and breast cancer basal versus mesenchymal. Of these 130 genes, 86 (66%) were down regulated during progression and 44 (34%) were up regulated. Notable up regulated genes included the FOX transcription factors FOXA2, FOXD1, and FOXQ1, Histones HIST1H2BD and HIST2H2AC, and the STAT1 transcription factor.

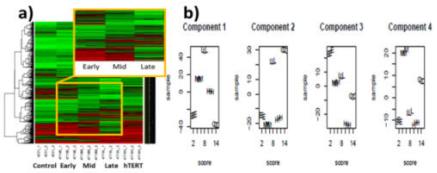

Figure 2: Gene Expression and Principle Component Analysis of Expression Data. a) unsupervised clustering of gene expression. Red is greater expression and green is less expression. hTERT-immortalized cells are similar to early passage cells, while spontaneously immortalized cells (Late) show gain of expression of a few genes, but loss of expression of two blocks of genes in the middle and bottom of the figure (highlighted on inset). b) Principle Component Analysis identified 4 key components. The first component shows the best separation between time points and the expected order of progression. C is Control, E is Early, M is Mid, L is Late (immortalized) and H is hTERT-immortalized.

CpG methylation

Unsupervised cluster analysis of the 1% most variable methylation probes (Figure 3) confirms that early passage HMEC 4231 and early passage HMEC PJ4719E are closely related. hTERT-immortalized PJ4719H cells are generally similar to the other early passage cells but have slightly more CpG island methylation and have begun to show some non-CpG island hypo-methylation.

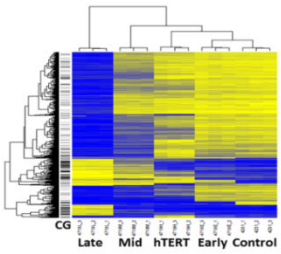

Figure 3: Unsupervised Cluster Analysis of DNA Methylation. Yellow is methylated and blue is not methylated. In the CG column, black denotes a CpG island and shades of gray correlate with density of CpG's in "shores" and "shelves".

Progression to spontaneous immortalization is characterized by striking global hypo-methylation and regional hyper-methylation of CpG islands. Methylation profiles at the various time points were compared with TCGA data (correlation) for normal breast tissue, breast cancer tissue, and breast cancer cell lines. Transition from early passage to immortal cells is characterized by decreasing identity with normal tissue and increasing identity with cancer cell lines (data not shown). Thirteen genes were common to the 130 gene list driving the gene expression PCA and the 1% most variable methylation markers. All 13 genes showed increased methylation with progression to immortalization and expression of 11 of these genes was down-regulated with progression. Themes that emerge from this short gene list include dys-regulation of retinoic acid signaling (CBS, RARRES1, and RBP1), reduced ubiquination of tyrosine kinases (CBLC, HERC5) and loss of signals that maintain epithelial differentiation (FBLN2, LAD1, OVOL2). Of note, CDKN2A (p16) was un-methylated in early passage cells, 50% methylated in PJ4719M mid-passage cells, and fully methylated in immortal PJ4719L cells, but did not occur on the list of 130 genes most relevant to the PCA.

DNA copy number

Immortal HMEC PJ4719L showed some evidence of focal amplifications and deletions across the genome (Figure 4a) but only deletions were statistically convincing.

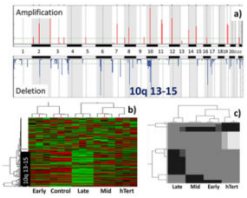

Figure 4: DNA Copy Number. a) Distributions of amplifications and deletions observed in immortalized HMEC 4719L cells. b) *mRNA* expression from amplified and deleted regions. Immortal HMEC 4719L show markedly reduced *mRNA* expression from the deleted 10q 13-15 region. c) Heat map of chromosomal regions with statistically significant amplification or deletion. Black is deleted, white is amplified and grey is diploid. There are 1,421 genes represented on this figure.

Most notable was a large deletion across 10q 13-15 in immortal PJ4719L cells that translated into significantly reduced mRNA expression from this region (Figure 4b). Early passage PJ4719E cells show deletion of 9q34.3 which is also evident in middle time point PJ4719M cells, but absent from spontaneously immortalized PJ4719L cells and hTERT-immortalized PJ4719H cells (Figure 4c). Middle passage cells acquired additional deletions at 9p13 and 11q22, but these were not carried forward into immortalized PJ4719L cells. Of note, hTERT immortalization was associated with amplification at 9q 21-22 and 9q31-33 which was supported by evidence of increased mRNA expression from this region.

The large deletion at 10q 13-15 evident in immortal PJ4719L cells did not contribute to the gene expression changes driving the PCA. DNA deletions were observed for only 4 of the 130 genes driving the PCA. Of these CCDC3 (10p), COL6A3 (2q), and NRARP (9q) were down-regulated and CD70 (19p) was up-regulated.

Micro-RNA

miR expression variance was calculated for PJ4719E, PJ4719M and PJ4719L cells. From 3,461 starting probes, 32 unique *miR* corresponded to the 1% most variable probes (supplemental Table 2). Sixteen of these were up-regulated and 16 down-regulated during progression to immortalization. Putative gene target lists were generated for each *miR* using miRDB [16], but none of the lists showed significant identity with the 130 genes whose expression changes best differentiated between the different time points and samples (Supplemental Table 1). The expression pattern of these 32 most variable *miR* for the HMEC 4231 control cells correlated best with the expression pattern of the PJ4719E cells (Spearman r=0.876, P <0.0001). hTERT-immortalized PJ4719H cells were most similar to PJ4719M cells (Spearman r=0.895, P <0.0001).

The 130 gene PCA list was also used as input for miRDB to identify *miR* that may be linked to expression of these genes. The *miR-200* family, including *200a-3p*, *200b-3p*, and *200c-3p*, was significantly down-regulated during progression to immortalization. This *miR* family is important in mammary epithelial cell differentiation [17] and suppression of epithelial-to-mesenchymal transition [18]. Potential target genes for *miR-200* from the 130 gene PCA list include *FBXO32, FN1, GUCT1A3, IL1A, LRAT, PRKAR1A, SIK1* and *TFPI.*

Exome sequencing

PJ4719E, PJ4719M and PJ4719L all showed an STK11 Chr19:1207168 C>T mutation (STK11 256C<T R86X). PJ4719M cells acquired 162 variants not seen in PJ4719E while PJ4719L acquire 214 variants not seen in PJ4719E. Only 9 new variants carried forward from PJ4719M to PJ4719L. Immortalized PJ4719L cells had 17 variants not seen in earlier passage cells (Supplemental Table 3). None of these genes occurred on the list of 130 genes whose expression most strongly influenced the PCA.

Discussion

Normal mammary epithelial cells have a very low rate of cell division *in vivo*, but when placed in culture, on plastic, they begin to divide rapidly. Growth promoting signals may include the stiff matrix provided by plastic and the epidermal growth factor included in the serum-free medium. The latter is likely responsible for the outgrowth of estrogen receptor-negative cells, as normal luminal mammary epithelial cells do not express epidermal growth factor receptor, but basal cells do. Some have argued that HMEC are an artifact of the *in vitro* culture conditions and not relevant to *in vivo* transformation of mammary epithelial cells. Data presented here suggest that spontaneously immortalizing HMEC is a reasonable representation of adaptive changes evoked by growth in a "stressed" environment. As such, they will reflect many of the same processes integral to malignant transformation *in vivo*. Indeed, the gene list derived from principle component analysis of changes in gene expression over the course of immortalization showed exceptional homology (adjusted P-values<10-20) with more than 100 published gene sets, most relevant to breast cancer. The molecular evolution of immortal HMEC described here cannot be dismissed as an artifact of in vitro culture conditions.

HMEC placed in culture undergo several doublings before encountering M0 growth arrest. The cause of this growth arrest is uncertain, but escape from M0 is traditionally attributed to silencing of the cell cycle control gene CDKN2A through promoter region methylation [19]. CDKN2A promoters were 50% methylated in PJ4719M immediate post-M0 cells and 100% methylated in PJ4719L immortal cells. However, escape from M0 growth arrest was associated with broader epigenetic reprogramming characterized by modest global hypo-methylation and regional hyper-methylation of several CpG islands (Figure 3). CDKN2A was among the 258 genes whose methylation was associated with escape from M0. This level of epigenetic remodeling during escape from M0 growth arrest has been observed previously [20].

Global hypo-methylation of non-coding DNA and regional hyper-methylation of promoter-associated CpG islands is a hallmark of epithelial malignancy [21], and was a central feature of immortalized PJ4719 HMEC. Global hypo-methylation largely affects highly repeated interspersed DNA sequences such as the satellite DNA of heterochromatin or the retro-transposable long interspersed element–1 (LINE-1) DNA [22]. Hypo-methylation of either of these DNA species can lead to genomic instability which increases genetic diversity in cell populations. This is potentially, an adaptive mechanism for surviving a stressful microenvironment. It is also possible that DNA hypo-methylation causes loss of imprinting resulting in double doses of growth factor gene products. In addition, some have suggested that DNA hypo-methylation can turn on "cancer testis antigens" (X-chromosome genes more frequently active in cancer) [23]. We did not see evidence for this as the X-chromosome was not over-represented among the genes that were up-regulated during PJ4719 immortalization. Conversely, cell line studies have found that DNA hypo-methylation is actually associated with repressive chromatin formation and gene silencing [24]. Whatever the biological effects, global DNA hypo-methylation has been recognized as a feature of epithelial malignancy and has been proposed as a marker in cell-free plasma for disease detection and monitoring [25]. Our results suggest that global DNA hypo-methylation is a feature of the immortalization phase of malignant transformation and may, therefore, first appear in high risk preneoplasia. Indeed, atypical hyperplasia shows the same degree of LINE-1 DNA hypo-methylation as in situ and invasive cancer [26], and there is also evidence of hypo-methylation in LCIS [27].

The second growth arrest, termed M1, has been attributed to telomere shortening which leads to genomic instability [20]. Escape from M0 has been related to expression of telomerase which stabilizes telomere length. Immortal PJ4719L cells exhibited more DNA amplifications, deletions and point mutations than their precursor

cells, but expression data did not convincingly show any change in telomerase expression. The DNA copy number data (Figure 4c) show that immortal PJ4719L cells do not share the same DNA deletions observed in the earlier passage cells. This suggests that the immortal clone arose from a minor cell population that was unrecognizable among the bulk post-MO cells.

Spontaneous immortalization of HMEC in culture is extremely rare. It is likely that these HMEC from a young Peutz-Jeghers breast cancer patient already harbored a "prepared" cell population that was somehow primed for malignant transformation. However, gene expression (Figure 2) and methylation (Figure 3) patterns are striking similar for the early passage PJ and control HMEC. GSEA analysis of the few gene expression differences does point to metabolism of proteins, translation, and metabolism of RNA as affected pathways which suggest that the mTOR pathway is affected by the heterozygous STK11 mutation. The *miRNA* array does show some differences between control and early passage PJ HMEC (Supplemental Figure 1), mostly up-regulation of several *miRNAs* in the PJ HMEC relative to the control cells. Among the greatest differences is up-regulation of the miR-199a/214 complex in the PJ4719E HMEC. The miR199a/214 cluster has been shown to reduce cell adhesion and increase migration by targeting claudin-2 and E-cadherin [28].

Because spontaneous immortalization is extremely rare, some investigators have studied early passage HMEC that have been immortalized by forced expression of hTERT. Though gene expression and *miRNA* profiles of hTERT-immortalized PJ4719H are similar to post-M0 PJ4719M cells, the hTERT-immortalized cells lack much of the global hypo-methylation and regional hyper-methylation seen in post-M0 cells. The CDKN2A promoter shows very little methylation in the hTERT-immortalized cells, making them more like early passage HMEC. In addition, hTERT immortalized cells have amplified several regions of DNA, a feature not seen in spontaneously immortalized HMEC. Because they lack the profound epigenetic reprogramming observed in spontaneously-immortalized cell, hTERT-immortalized HMEC may not be representative of transforming breast epithelial cells.

Progression of normal HMEC to immortalized cells is characterized by sequential changes in expression of a discrete collection of genes. The gene expression changes mirror those seen in primary breast cancer establishing the relevance of these in vitro observations. Some of the key gene expression changes are directly attributable to observed promoter region methylation, changes in *miRNA* expression, and DNA deletion, amplification, or mutation. Epigenetic reprogramming is a central feature of immortalization as evidenced by the observed global hypo-methylation and regional hyper-methylation. Immortalized HMEC are not cancer cells as they are incapable of anchorage-independent growth and do not form tumors in immune-compromised mice. High risk preneoplasia and ductal carcinoma in situ may be the most similar counterparts in nature.

Conclusions

Immortalization may result from adaptive changes evoked by growth in a "stressed" environment. Epigenetic reprogramming rather than mutation is a central feature that may be exploitable for tissue-based breast cancer risk stratification and management.

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