Low Expression of Estrogen Receptor-α and Progesterone Receptor in Human Breast Cancer Tissues Is Associated with High-grade Human Cytomegalovirus Protein Expression

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MicroAbstract

The mechanisms involved in initiation and progression of breast cancer (BC), the most common malignancy and second leading cause of cancer deaths in women, are largely unknown. Human cytomegalovirus (HCMV) has been detected in primary BC, sentinel lymph nodes and brain metastases of BC patients. We found HCMV DNA, RNA, and proteins in BC, and their prevalence was higher in advanced cancer. HCMV levels correlated inversely with estrogen (*P = 0.02*) and progesterone (*p = 0.003*) receptor expression. HER2 expression was also found to be decreased in HCMV-positive samples without reaching a level of statistical significance (*p = 0.09*). Our findings showing that high-grade expression of HCMV immediate-early protein correlated negatively with hormone receptor expression, suggest a role for HCMV in the development of hormone-receptor-negative BC in subgroups of patients, possibly by hampering hormone receptor expression and forcing BC cells into a more aggressive and hormone-independent phenotype.

Key words: human cytomegalovirus, breast cancer, estrogen receptor-α, progesterone receptor, human epidermal growth factor receptor 2.

Abstract

Background: The underlying mechanisms for breast cancer (BC) are largely unknown. We investigated possible correlations between the expression levels of human cytomegalovirus (HCMV) proteins and established histopathological markers of BC, including expression of estrogen receptor-α (ER- α), the progesterone receptor (PgR), and human epidermal growth factor receptor-2 (HER2).

Material and Methods: We retrospectively examined paraffin-embedded biopsy specimens of BC (*n* = 62), ductal carcinoma in situ (*n* = 19), and adjacent normal breast tissue (*n* = 42) for HCMV immediate-early protein (IE), HCMV late antigen, HCMV DNA and RNA, and investigated possible correlations between them and expression of ER-α, PgR, and HER2.

Results: HCMV DNA and RNA were detected in all examined infiltrating BCs. High-grade positivity for HCMV-IE was detected in 77% of infiltrating BCs, 39% of ductal carcinomas in situ, and 7% of tumor-free breast tissue samples. HCMV expression correlated inversely with ER- α (*p = 0.02*) and PgR (*p = 0.003*) expression. HER2 expression was also reduced in HCMV-positive samples without reaching a level of statistical significance (*P = 0.09*).

Conclusion: The negative correlation between high-grade expression HCMV-IE and hormone receptor expression suggests a role for HCMV in hormone-receptor-negative BC tumors, possibly by forcing BC cells into a more aggressive phenotype.

Introduction

Breast cancer (BC) is the most common type of malignancy and the second leading cause of cancer deaths in women worldwide. Its prevalence differs considerably by geographical region, suggesting that environmental factors are important in its development [1]. Risk factors include genetic aberrations such as mutations in *BRCA1* and *BRCA2*; endocrine factors such as pregnancies, breast feeding, age at menarche and menopause, and hormone-replacement therapy; and life-style factors such as exercise, nutrition, and body mass index [1]. However, despite improved knowledge of its molecular biology, BC is steadily increasing in prevalence worldwide, and a cure is lacking for metastatic disease [2, 3]. Targeted therapies used as adjuvant therapy for subgroups of patients, such as those whose tumors express human epidermal growth factor receptor 2 (HER2), have increased the chance for a cure in some patients and have prolonged progression-free survival in patients with metastatic disease. Unfortunately, fatal metastatic disease still develops in many patients [4-6].

Only some of the poor outcomes in patients with metastatic disease can be explained by tumor heterogeneity and plasticity or by many mechanisms of drug resistance [7-10]. The most important predictive factors to guide therapy are the expression levels of the estrogen receptor (ER), the progesterone receptor (PgR), and HER2 [11, 12]. These factors and the proliferation marker Ki-67 are used to classify BC into four mainly descriptive subgroups: luminal-A, luminal-B, HER-2-positive, and triple-negative (TNBC), the most aggressive form. As many as 90–95% of all BCs are sporadic, indicating that the mechanisms are unknown [13]. Therefore, it is important to identify alternative factors that may initiate and promote BC.

Human cytomegalovirus (HCMV) has been detected in glioblastoma multiforme [14, 15], medulloblastoma [16], neuroblastoma [17], colon cancer [18, 19], and prostate cancer [20], as well as in primary BC and sentinel lymph nodes and distant metastases from BC patients [21-23]. In general, increased viral activity is associated with worse prognosis. In xenograph models, antiviral treatment of HCMV-positive tumors reduces tumor growth [16, 17]. We showed that treating glioblastoma patients with valganciclovir for more than 6 months prolongs overall survival [24, 25]. Thus, these findings support the hypothesis that HCMV may contribute to cancer

development or progression, and antiviral therapy may be a new way to target HCMV-positive cancers, including selected BC patients.

HCMV, a member of the herpes viridae family, is common in populations worldwide. In humans, the virus can escape immune elimination and establish latency in myeloid lineage cells [26]. During inflammation, latent infections may be re-activated [27], leading to production of new viral particles and release of factors that can enhance inflammation and damage surrounding tissues. HCMV infects many cell types, including epithelial cells, endothelial cells, and fibroblasts. The virus infection causes the intracellular synthesis of some 750 proteins; many are relevant to tumor biology, and some may highjack the host cell machinery [28-30]. Although HCMV is found in many tumor types, can affect most hallmarks of cancer [31-34], and under certain circumstances induces oncogenic transformation [35, 36], it is not classified as an oncogenic virus. Instead, it is currently described as "oncomodulatory", referring to its ability to influence tumor cell behavior and progression, for example by controlling host cell gene expression and intra-cellular signaling molecules that contribute to cancer biology, including p53, Rb, cyclins, Wnt, PI3K/Akt, and NF-κB [31-34, 37, 38].

In this study, we investigated the prevalence of HCMV in samples of infiltrating BC, ductal carcinoma in situ (DCIS), and adjacent normal breast tissue. We also investigated possible correlations between the expression of HCMV proteins and established histopathological markers in human BC tissues, such as ER, PgR, and HER2 expression, the Ki-67 labeling index, nodal status, tumor size and extension into neighboring breast tissue (tumor stadium), and histologic grade.

Materials and Methods

Study design

Paraffin-embedded samples of infiltrated BC (n = 62), DCIS (n = 19), and adjacent normal breast tissue (n = 42) were retrospectively obtained from 62 patients who underwent surgery at Akershus University Hospital, Oslo, Norway during 2011. Baseline clinical data (Table 1) were provided by the Departments of Oncology and Pathology at Akershus University Hospital. All

diagnoses were re-confirmed by an experienced BC pathologist (T.S.) at our hospital. The median age at surgery was 55 years. Most patients (60%) underwent mastectomy; 22 (36%) had breast-conserving surgery, and 5% had bilateral surgery. All patients received standard adjuvant treatment according to Norwegian guidelines (Table 1).

All patients gave informed consent. The study protocol was approved by the ethical committee at the Karolinska Institutet (Dnr: 2008/628-31/2), Stockholm, Sweden, and by the regional ethical committee of south-east Norway, Oslo, Norway (577-06-04148, 06118).

Immunohistochemistry

Tissue microarrays were created, and all tissues were sectioned (4 μm) and analyzed by immunohistochemical techniques optimized in our laboratory. Briefly, tissue sections were deparaffinized in xylene (Sigma Aldrich) and rehydrated in an ethanol series (Apoteket Farmaci). Immunohistochemical staining for HCMV proteins was done as described previously with minor modifications [21]. Tissues were deparaffinized and rehydrated and washed in Tris-buffered saline, pH 7.5, containing Triton X-100 (Substrate Department, Karolinska University Hospital). Antigen retrieval and unmasking were done by heating the tissues in DIVA decloaker buffer (Histolab), pH 6.2, in a pressure cooker (BioCARE) for 15 min. Endogenous peroxidase activity was blocked with peroxidase 1 (Histolab) for 5 min, and nonspecific binding was blocked with Sniper (Histolab) for 16 min at room temperature. The tissue sections were then incubated with antibodies against HCMV-IE and HCMV-LA (Ca# MAB810R, MAB8127, IgG2a, Merck Millipore), ER-α (Ca# M3643, Clone EP1, IgG, Dako, Denmark), PgR (Ca# SC-539, Clone C10, IgG, Santa Cruz Biotechnology), HER2 (Ca# MA1-90362, Clone 3B5, IgG1, Thermo Fisher Scientific), cytokeratins 5, 6, 8, 17, and 19 (Ca# M0821, Clone MNF116, IgG1, Dako), and von Willebrand factor (Ca# M0616, Clone F8/86 IgG1, Dako). Antibodies against cytokeratin 20 (Ca# K0199-25, IgG2a, US Biological) served as negative control.

HCMV staining was evaluated as described [21], according to the estimated percentage of cells expressing HCMV proteins (IE, LA): negative (0%), grade 1 (<25%), grade 2 (≥25–50%), grade 3 (≥50–75%), and grade 4 (≥75%). To ensure a representative number of patients in each

category for statistical analysis, tumors were considered as HCMV-negative or as having low-grade HCMV infection (<50% positive cells) or high-grade infection (≥50% positive cells). Immunohistochemical staining for HCMV was evaluated and graded by a senior scientist (A.R.) who was blinded for the clinical records. Immunohistochemical staining for ER-α, PgR, HER2, and Ki-67 was done and evaluated at the Department of Pathology at Akershus University Hospital.

In situ hybridization

Paraffin-embedded BCs from 23 patients were examined for HCMV-DNA and RNA by in situ hybridization. Tissue sections were deparaffinized in xylene (Sigma), dehydrated in ethanol (Apoteket Farmaci), air dried, and pretreated with Vysis paraffin pretreatment IV (CA# 01N31-005, Abbott) as recommended by the manufacturer. The HCMV genome was detected with a digoxigenin-labeled CMV probe that recognizes HCMV-DNA and β2.7 RNA (CA# RI0011T, Biocare Medical). A probe recognizing Alu repeat sequences (CA# BRR4026T, Biocare Medical) and a probe consisting of a random set of oligonucleotide sequences were used as positive control and negative controls, respectively. Digoxigenin-labeled probes in the tissues were detected with Zytofast Plus CISH Implementation Kit AP-NBT-BCIP (CA# T-1061-40, ZytoVision).

Statistical analysis

All analyses were done with GraphPad Prism; $p < 0.05$ was considered statistically significant. Chi-square and Fisher's exact tests were used to assess the statistical significance of associations between HCMV infection levels and hormone receptor expression levels and clinical factors. Differences in categorical variables were assessed with the chi-square test, Fisher's exact test and Mann Whitney test.

Results

High-grade HCMV-IE positivity is common in BC and DCIS but rare in normal breast tissue

Representative photomicrographs of sections immunostained for HCMV-IE, HCMV-LA, and cytokeratins are shown in Fig. 1. During staining, a few tissues sections were lost (detached from the slides). The numbers of samples available for staining and the results are shown in Figure 2. HCMV-IE was detected in all examined tissue sections and HCMV-LA was detected in 75% of BC, 47% of DCIS and in 68% non-tumor tissue sections at different levels (Fig. 2A). Significantly higher number of patients with BC were positive for HCMV-LA in their tumor compared to those with DCIS ($p= 0.01$) (Fig. 2A). In 51% of the patients with infiltrating BC, >75% of the cells within the tumor expressed high grade HCMV-IE compared to only 22% in DCIS ($p= 0.002$) and 5% in adjacent non tumor tissues ($p <0.0001$) (Fig. 2B).

In infiltrating BC, HCMV-IE expression was present in 53 samples and at high grade (grade 3 or 4) in 77% compared to 39% in DCIS (grade 3 or 4) ($p= 0.004$) and in 7% adjacent non tumor tissues (grade 3 or 4) ($P <0.0001$) (Fig. 2C). HCMV-LA expression was absent in 25% and in the remaining ones was more often low grade than high grade (72% vs. 3%). In 18 DCIS samples, HCMV-IE expression was more often low grade than high grade (61% vs. 39%), whereas in 19 DCIS samples, HCMV-LA expression was absent in 53% and in the others was more often low grade than high grade (26% vs. 11%). In 42 normal breast tissue samples, HCMV-IE expression was present in all of them and was more often low grade than high grade (93% vs. 7%), whereas in 40 samples, HCMV-LA expression was absent in 33% and in the others was more often low grade than high grade (63% vs. 5%) (Fig.2B-C). Cytokeratins 5, 6, 8, 17, and 19 were detected in all samples, and cytokeratin 20 was not detected in any of them.

All BCs were positive for HCMV DNA and β2.7 RNA by in situ hybridization

Digoxigenin-labeled probes recognizing HCMV-DNA and β2.7 RNA detected the HCMV genome in all 23 BC samples examined by in situ hybridization. No signal was detected in

negative controls while the housekeeping gene *Alu* (positive control) was detected in all of them.

High-grade HCMV-IE expression is associated with low levels of ER-α expression in BC

Data on ER-α expression in infiltrating BCs were available for 47 patients (Fig. 3A, B). High-grade HCMV-IE expression was associated with low levels of ER-α expression in BC tissues ($p = 0.02$) (Fig. 3A). High-grade HCMV-IE expression was detected in 100%, 86%, and 74% of patients whose tumors had 0–10%, >10–50%, and >50–100% ER-expressing cells, respectively. The corresponding percentages for patients with low-grade HCMV-IE expression were 0%, 14%, and 26%. ER-α expression was decreased or absent in tumors in which HCMV-IE was expressed predominantly in the nucleus (Fig. 3A and B).

High-grade HCMV-IE expression is associated with low levels of PgR expression in BC

Data on PgR expression in infiltrating BCs were available for 44 patients (Fig. 4A, B). High-grade HCMV-IE expression was associated with low levels of PgR expression ($p = 0.003$) (Fig. 4A). High-grade HCMV-IE was detected in 83%, 76%, and 40% of patients whose tumors had <10%, >10–50%, and >50–90% PgR-expressing cells, respectively. The corresponding percentages for patients with low-grade HCMV-IE expression were 17%, 24%, and 60% (Fig. A).

High-grade HCMV-IE is not significantly associated with HER2 expression

Clinical data on HER2 expression in BC samples analyzed by immunohistochemistry and fluorescence in situ hybridization (FISH) was available for 53 patients. Most of the tumors were HER2 negative (81%). In HER2-negative tumors, HCMV-IE expression was high-grade in 68% and low-grade in 13% (Fig. 5A). In HER2-positive tumors, HCMV-IE expression was high grade in 15% and low grade in 4% (Fig. 5A). HER2 expression was not statistically significant associated with high-grade HCMV-IE expression in BCs although a clear trend was observed ($p = 0.09$) (Fig. 5A and B).

HCMV-IE positivity is not associated with other clinical markers of BC except for histopathological grade

Data on the Ki-67 labeling index, nodal status, tumor stadium, histological grade, and menopause status are shown in Table 1. High-grade HCMV-IE expression was detected in 71% and 82% of patients with >1–30% and >30–80% Ki-67-positive cells in their tumor, respectively. HCMV-IE expression was not associated with the Ki-67 labeling index ($p= 0.5$, Supplementary Fig. S1A). The corresponding percentages for patients with low-grade HCMV-IE expression in their tumors were 32% and 18%. High-grade HCMV-IE expression was found in tumors of 75%, 91%, and 60% of patients with nodal status of N0, N1, and N2-3, respectively (Supplementary Fig. S1B). Slightly more patients with N1 had high-grade HCMV-IE than patients with N0. No significant difference was detected between high grade HCMV-IE (>50% positive cells within the tumor) in patients with status N0 vs N1 ($p= 0.4$), N0 vs N2-3 ($p= 0.4$) or N1 vs N2-3 (p= 0.15)(Supplementary Fig. S1B). High-grade HCMV-IE expression was detected in similar tumors of patients with tumor stadium 1 (71%) and 2 (80%) ($p= 0.5$) (Supplementary Fig. S1C). Moreover, 88% of patients with premenopausal and perimenopausal (n = 17 and n=8, respectively) and 63% of postmenopausal patients (n = 27) had high-grade HCMV-IE expression in their tumors ($p= 0.9$). No statistical significant differences was detected in the number of patients having high grade HCMV-IE in their tumor at premenopausal vs perimenopausal ($p= 0.9$), premenopausal vs postmenopausal ($p= 0.9$) or perimenipausal vs postmenopausal ($p=0.2$) (Supplementary Fig. S1D). Finally, significantly higher number of patients with infiltrating ductal cancer (n=35, 81.4%) had high-grade HCMV-IE expression in their tumors compared to 50% of patients (n=5) with infiltrating lobular cancer, medullar cancer, multiple caner types or mucinous subtype ($p= 0.03$) (Supplementary Fig. S1E).

Discussion

In this study, we detected HCMV proteins and nucleic acids in all BC specimens, consistent with previous reports [21, 25, 39]. HCMV-IE protein was present in all infiltrating BC, DCIS, and adjacent normal breast tissues, whereas HCMV-LA expression was absent in 25% of infiltrating BC, 53% of DCIS, and 33% of adjacent normal tissue. High-grade HCMV-IE expression (i.e., >50% of tumor cells) was detected in 77% of infiltrated BC, but in only 39% of DCIS and 7% of adjacent normal tissue, and high-grade HCMV-LA was detected in only 3%, 11%, and 5%, respectively. Cytokeratins 5, 6, 8, 17, and 19, which are markers for classifying neoplastic cells of epithelial origin, were found in all samples of BC, DCIS, and adjacent normal tissue, suggesting ongoing cellular activity favoring neoplastic transformation of epithelial cells, even in adjacent normal breast tissue, leading to induced expression of these cytokeratins.

To further explore possible oncomodulatory effects of HCMV in BC, we sought to determine whether HCMV-IE expression correlated with ER-α, PgR, or HER2 expression, menopause status, Ki-67 labeling index, histopathological grade, or TNB classification. High-grade HCMV-IE expression was significantly associated only with low-level expression of ER-α ($p = 0.02$) and PgR ($p = 0.003$). Notably, although HER2 levels were also reduced in tumors with high-level expression of HCMV-IE, the correlation was not significant ($p = 0.09$). Taken together, these observations suggest that HCMV infections may influence significantly on the BC phenotype by forcing tumor cells in the direction of a triple-negative phenotype (TNBC).

Approximately 10–15% of BCs are classified as TNBC, for which treatment options are very limited [40]. Out of 10 TBNC cases in our study, 7 (70%) had high-grade expression of HCMV-IE. TNBC is characterized by negativity for ER-α, PgR, and HER2. In addition, TNBC is characterized by p53 mutations, and overexpression of cytokeratins 5/6 and 14/17, caveolins 1 and 2, cyclin-D1, and p-cadherin [41-43]. Hormone receptor and HER-2 expression in TNBC are lost through mechanisms largely unknown. Although TNBC can be associated with germline *BRCA1* mutations [44-46], only 10% of TNBC are associated only with mutations in *BRCA1* [44, 45]. Furthermore, gene analysis showed that TNBC has a defined molecular signature and is regulated by the angiotensinogen molecular network, NF-κB, platelet-derived growth factor

receptor, and p53 pathways, which are important in angiogenesis, cell-cycle regulation, and inflammation [47].

Several microRNAs (miRNAs) have also been associated with TNBC. Some are normally activated by p53 and can reduce the stability and translation of ER-α transcript [48]. miR-22, miR-206, miR-221, and miR-222 can reduce ER-α expression [49], and *miR-520g* expression is elevated in ER- and PgR-negative tumors, indicating a role for these miRNAs in the regulation of steroid receptor and growth factor expression and TNBC development [50]. In addition, epigenetic modulation may also contribute to the development of TNBC. Hypermethylation of ESR1 is increased in BC patients [51], and ER-α expression can be restored in ER-α-negative BC cells by inactivating DNMT-1 with 5-azadeoxycytidine or siRNA [52]. Furthermore, a study comparing ER/HER2-negative and ER/HER2-positive BCs showed frequent hypermethylation of the genes encoding adenomatous polyposis coli, glutathione S-transferase P1, RASSF1A (a putative tumor suppressor), and TWIST (a human basic helix-loop-helix DNA binding protein) and suggested a possible role for epigenetic regulation in silencing ER and HER2 expression [53].

Furthermore, high grade HCMV-IE was significantly more prevalent in patients with infiltrating ductal cancer compare to those with infiltrating lobular cancer, medullar cancer, multiple cancer types or mucinous subtype. All patients who died due to their breast cancer diagnosis in our study (n=4) were diagnosed with infiltrating ductal cancer and they had high grade HCMV-IE in their tumors. This finding may further strengthen the oncomodulatory role of HCMV in BC towards a poor prognosis for the patients.

Presence of HCMV proteins and nucleic acids in BC might be due to migration and reactivation of latent HCMV due to existing inflammation in tumor microenvironment. In fact, inflammation has been strongly associated with tumor development and is believed to drive reactivation of latent HCMV [26, 27]. In theory, oncogenic activity and transformation of latently HCMV infected cells might lead to reactivation of virus.

HCMV-IE proteins are viral regulatory proteins that act as transcription factors. Their oncomodulatory effects reflect their ability to interfere with p53 and pRb protein family members, which leads to increased proliferation [54] by promoting entry into the S phase of the

cell cycle [32, 37]. HCMV-IE proteins also cause chromosome instability [55], induce expression of proto-oncogenes, cyclins, and kinases involved in cell division [31], and activate cellular signaling pathways to increase survival by activating the transcription factor NF-κB [33, 38]. Expression of IE proteins together with adenovirus E1A protein results in cellular transformation [36]. Furthermore, HCMV alters epigenetic patterns in infected cells [56] and produces 26 viral encoded miRNAs that can affect cellular functions [57]. HCMV produces six anti-apoptotic proteins, two of which mediate resistance to chemotherapy [58, 59]. Hence, HCMV IE proteins may contribute to BC progression and tumor response to therapy.

In conclusion, our study is the first to show a significant association between HCMV-IE protein expression and hormone receptor expression in BC. A higher grade of HCMV activity was associated with loss of expression of ER-α, PgR, and potentially HER2—a signature of TNBC, one of the most aggressive BC subtypes. It is hence possible that the expression levels of the prognostic markers ER-α, PgR, and HER2 reflect HCMV activity in tumors and that HCMV activity directly contributes to tumor aggressiveness. Given the prognostic value of hormone receptor status, a better understanding of the mechanisms by which HCMV downregulates hormone receptor expression in BCs could be of high clinical relevance. Furthermore, as TNBC patients have a poor prognosis with limited treatment options, antiviral therapies may be of value as add-on to standard therapies for carefully selected patients and should hence be evaluated in future clinical trials.

Author contributions

Designed the study: A. Rahbar, C. Soderberg-Naucler, K. Vetvik, J. Geisler, and I. R. Buckholm.

Provided all clinical data: T. Sauer, J. Geisler, J. Touma.

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Evaluated immnohistochemistry and in situ hybridization: A. Rahbar.

Evaluated the pathological diagnosis: T. Sauer.

Analyzed data and performed statistical analysis: A. Rahbar.

Helped to interpret the data: A. Rahbar, C. Soderberg-Naucler, K. Vetvik, J. Geisler, H. Costa, and J. Touma.

Wrote the manuscript: A. Rahbar, C. Soderberg-Naucler, K. Vetvik, and J. Geisler.

All authors reviewed the manuscript, provided comments, and approved the final version of the manuscript.

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References

1. McPherson K, Steel CM, Dixon JM. ABC of breast diseases. Breast cancer-epidemiology, risk factors, and genetics. *BMJ.* 2000;321:624-628.
2. Brown SB, Hankinson SE. Endogenous estrogens and the risk of breast, endometrial, and ovarian cancers. *Steroids.* 2015;99:8-10.
3. Falconer H, Yin L, Gronberg H, Altman D. Ovarian cancer risk after salpingectomy: a nationwide population-based study. *J Natl Cancer Inst.* 2015;107.
4. Perou CM, Sorlie T, Eisen MB, et al. Molecular portraits of human breast tumours. *Nature.* 2000;406:747-752.
5. Pusztai L, Ayers M, Stec J, et al. Gene expression profiles obtained from fine-needle aspirations of breast cancer reliably identify routine prognostic markers and reveal large-scale molecular differences between estrogen-negative and estrogen-positive tumors. *Clin Cancer Res.* 2003;9:2406-2415.
6. Gianni L, Zambetti M, Clark K, et al. Gene expression profiles in paraffin-embedded core biopsy tissue predict response to chemotherapy in women with locally advanced breast cancer. *J Clin Oncol.* 2005;23:7265-7277.
7. Liu G, Yuan X, Zeng Z, et al. Analysis of gene expression and chemoresistance of CD133+ cancer stem cells in glioblastoma. *Mol Cancer.* 2006;5:67.
8. Ishikawa F, Yoshida S, Saito Y, et al. Chemotherapy-resistant human AML stem cells home to and engraft within the bone-marrow endosteal region. *Nat Biotechnol.* 2007;25:1315-1321.
9. Li X, Lewis MT, Huang J, et al. Intrinsic resistance of tumorigenic breast cancer cells to chemotherapy. *J Natl Cancer Inst.* 2008;100:672-679.
10. Mego M, Mani SA, Cristofanilli M. Molecular mechanisms of metastasis in breast cancer--clinical applications. *Nat Rev Clin Oncol.* 2010;7:693-701.
11. Hayes DF. Prognostic and predictive factors revisited. *Breast.* 2005;14:493-499.
12. Hortobagyi GN, Smith TL, Legha SS, et al. Multivariate analysis of prognostic factors in metastatic breast cancer. *J Clin Oncol.* 1983;1:776-786.
13. Anders CK, Carey LA. Biology, metastatic patterns, and treatment of patients with triple-negative breast cancer. *Clin Breast Cancer.* 2009;9 Suppl 2:S73-81.
14. Rahbar A, Orrego A, Peredo I, et al. Human cytomegalovirus infection levels in glioblastoma multiforme are of prognostic value for survival. *J Clin Virol.* 2013;57:36-42.
15. Cobbs CS, Harkins L, Samanta M, et al. Human cytomegalovirus infection and expression in human malignant glioma. *Cancer Res.* 2002;62:3347-3350.
16. Baryawno N, Rahbar A, Wolmer-Solberg N, et al. Detection of human cytomegalovirus in medulloblastomas reveals a potential therapeutic target. *J Clin Invest.* 2011;121:4043-4055.
17. Wolmer-Solberg N, Baryawno N, Rahbar A, et al. Frequent detection of human cytomegalovirus in neuroblastoma: a novel therapeutic target? *Int J Cancer.* 2013;133:2351-2361.
18. Tafvizi F, Fard ZT. Detection of human cytomegalovirus in patients with colorectal cancer by nested-PCR. *Asian Pac J Cancer Prev.* 2014;15:1453-1457.
19. Dimberg J, Hong TT, Skarstedt M, Lofgren S, Zar N, Matussek A. Detection of cytomegalovirus DNA in colorectal tissue from Swedish and Vietnamese patients with colorectal cancer. *Anticancer Res.* 2013;33:4947-4950.
20. Samanta M, Harkins L, Klemm K, Britt WJ, Cobbs CS. High prevalence of human cytomegalovirus in prostatic intraepithelial neoplasia and prostatic carcinoma. *J Urol.* 2003;170:998-1002.
21. Taher C, de Boniface J, Mohammad AA, et al. High prevalence of human cytomegalovirus proteins and nucleic acids in primary breast cancer and metastatic sentinel lymph nodes. *PLoS One.* 2013;8:e56795.
22. Cox B, Richardson A, Graham P, Gislefoss RE, Jellum E, Rollag H. Breast cancer, cytomegalovirus and Epstein-Barr virus: a nested case-control study. *Br J Cancer.* 2010;102:1665-1669.

23. Harkins LE, Matlaf LA, Soroceanu L, et al. Detection of human cytomegalovirus in normal and neoplastic breast epithelium. *Herpesviridae.* 2010;1:8.

24. Soderberg-Naucler C, Rahbar A, Stragliotto G. Survival in patients with glioblastoma receiving valganciclovir. *N Engl J Med.* 2013;369:985-986.

25. Stragliotto G, Rahbar A, Solberg NW, et al. Effects of valganciclovir as an add-on therapy in patients with cytomegalovirus-positive glioblastoma: a randomized, double-blind, hypothesis-generating study. *Int J Cancer.* 2013;133:1204-1213.

26. Wills MR, Poole E, Lau B, Krishna B, Sinclair JH. The immunology of human cytomegalovirus latency: could latent infection be cleared by novel immunotherapeutic strategies? *Cell Mol Immunol.* 2015;12:128-138.

27. Soderberg-Naucler C, Fish KN, Nelson JA. Reactivation of latent human cytomegalovirus by allogeneic stimulation of blood cells from healthy donors. *Cell.* 1997;91:119-126.

28. Varani S, Landini MP. Cytomegalovirus-induced immunopathology and its clinical consequences. *Herpesviridae.* 2011;2:6.

29. Soderberg-Naucler C. Does cytomegalovirus play a causative role in the development of various inflammatory diseases and cancer? *J Intern Med.* 2006;259:219-246.

30. Stern-Ginossar N, Weisburd B, Michalski A, et al. Decoding human cytomegalovirus. *Science.* 2012;338:1088-1093.

31. Salvant BS, Fortunato EA, Spector DH. Cell cycle dysregulation by human cytomegalovirus: influence of the cell cycle phase at the time of infection and effects on cyclin transcription. *J Virol.* 1998;72:3729-3741.

32. Prichard MN, Sztul E, Daily SL, et al. Human cytomegalovirus UL97 kinase activity is required for the hyperphosphorylation of retinoblastoma protein and inhibits the formation of nuclear aggresomes. *J Virol.* 2008;82:5054-5067.

33. Yurochko AD, Kowalik TF, Huong SM, Huang ES. Human cytomegalovirus upregulates NF-kappa B activity by transactivating the NF-kappa B p105/p50 and p65 promoters. *J Virol.* 1995;69:5391-5400.

34. Cinatl J, Jr., Vogel JU, Kotchetkov R, Wilhelm Doerr H. Oncomodulatory signals by regulatory proteins encoded by human cytomegalovirus: a novel role for viral infection in tumor progression. *FEMS Microbiol Rev.* 2004;28:59-77.

35. Geder L, Sanford EJ, Rohner TJ, Rapp F. Cytomegalovirus and cancer of the prostate: in vitro transformation of human cells. *Cancer Treat Rep.* 1977;61:139-146.

36. Shen Y, Zhu H, Shenk T. Human cytomagalovirus IE1 and IE2 proteins are mutagenic and mediate "hit-and-run" oncogenic transformation in cooperation with the adenovirus E1A proteins. *Proc Natl Acad Sci U S A.* 1997;94:3341-3345.

37. Herbein G, Kumar A. The oncogenic potential of human cytomegalovirus and breast cancer. *Front Oncol.* 2014;4:230.

38. Khan KA, Coaquette A, Davrinche C, Herbein G. Bcl-3-regulated transcription from major immediate-early promoter of human cytomegalovirus in monocyte-derived macrophages. *J Immunol.* 2009;182:7784-7794.

39. Taher C, Frisk G, Fuentes S, et al. High prevalence of human cytomegalovirus in brain metastases of patients with primary breast and colorectal cancers. *Transl Oncol.* 2014;7:732-740.

40. Gordon V, Banerji S. Molecular pathways: PI3K pathway targets in triple-negative breast cancers. *Clin Cancer Res.* 2013;19:3738-3744.

41. Perou CM. Molecular stratification of triple-negative breast cancers. *Oncologist.* 2010;15 Suppl 5:39-48.

42. Oakman C, Viale G, Di Leo A. Management of triple negative breast cancer. *Breast.* 2010;19:312-321.

43. Bertucci F, Finetti P, Cervera N, et al. How basal are triple-negative breast cancers? *Int J Cancer.* 2008;123:236-240.

44. Foulkes WD, Stefansson IM, Chappuis PO, et al. Germline BRCA1 mutations and a basal epithelial phenotype in breast cancer. *J Natl Cancer Inst.* 2003;95:1482-1485.

45. Lakhani SR, Reis-Filho JS, Fulford L, et al. Prediction of BRCA1 status in patients with breast cancer using estrogen receptor and basal phenotype. *Clin Cancer Res.* 2005;11:5175-5180.

46. Chacon RD, Costanzo MV. Triple-negative breast cancer. *Breast Cancer Res.* 2010;12 Suppl 2:S3.

47. Ossovskaya V, Wang Y, Budoff A, et al. Exploring molecular pathways of triple-negative breast cancer. *Genes Cancer.* 2011;2:870-879.

48. Gyparaki MT, Basdra EK, Papavassiliou AG. MicroRNAs as regulatory elements in triple negative breast cancer. *Cancer Lett.* 2014;354:1-4.

49. Adams BD, Furneaux H, White BA. The micro-ribonucleic acid (miRNA) miR-206 targets the human estrogen receptor-alpha (ERalpha) and represses ERalpha messenger RNA and protein expression in breast cancer cell lines. *Mol Endocrinol.* 2007;21:1132-1147.

50. Lowery AJ, Miller N, Devaney A, et al. MicroRNA signatures predict oestrogen receptor, progesterone receptor and HER2/neu receptor status in breast cancer. *Breast Cancer Res.* 2009;11:R27.

51. Prabhu JS, Wahi K, Korlimarla A, et al. The epigenetic silencing of the estrogen receptor (ER) by hypermethylation of the ESR1 promoter is seen predominantly in triple-negative breast cancers in Indian women. *Tumour Biol.* 2012;33:315-323.

52. Yang X, Phillips DL, Ferguson AT, Nelson WG, Herman JG, Davidson NE. Synergistic activation of functional estrogen receptor (ER)-alpha by DNA methyltransferase and histone deacetylase inhibition in human ER-alpha-negative breast cancer cells. *Cancer Res.* 2001;61:7025-7029.

53. Sunami E, Shinozaki M, Sim MS, et al. Estrogen receptor and HER2/neu status affect epigenetic differences of tumor-related genes in primary breast tumors. *Breast Cancer Res.* 2008;10:R46.

54. Castillo JP, Kowalik TF. Human cytomegalovirus immediate early proteins and cell growth control. *Gene.* 2002;290:19-34.

55. Siew VK, Duh CY, Wang SK. Human cytomegalovirus UL76 induces chromosome aberrations. *J Biomed Sci.* 2009;16:107.

56. Esteki-Zadeh A, Karimi M, Straat K, et al. Human cytomegalovirus infection is sensitive to the host cell DNA methylation state and alters global DNA methylation capacity. *Epigenetics.* 2012;7:585-593.

57. Hook L, Hancock M, Landais I, Grabski R, Britt W, Nelson JA. Cytomegalovirus microRNAs. *Curr Opin Virol.* 2014;7:40-46.

58. Terhune S, Torigoi E, Moorman N, et al. Human cytomegalovirus UL38 protein blocks apoptosis. *J Virol.* 2007;81:3109-3123.

59. Poncet D, Pauleau AL, Szabadkai G, et al. Cytopathic effects of the cytomegalovirus-encoded apoptosis inhibitory protein vMIA. *J Cell Biol.* 2006;174:985-996.

Figure Legends

Figure 1. Detection of HCMV-IE and HCMV-LA in infiltrated BC, DCIS, and adjacent normal breast tissue by immunohistochemical staining (IHC) and in situ hybridization (ISH). (A) High-grade HCMV-IE expression and low-grade HCMV-LA expression in infiltrated BC specimens. (B) expression of HCMV-IE and HCMV-LA in adjacent normal breast tissues. Cytokeratin 5, 6, 8, 17, and 19 and served as positive staining controls, and keratin 20 served as a negative control. (C) Detection of HCMV-DNA and β2.7 RNA and the housekeeping gene Alu in BC. No signal was detected in negative controls. Lower panels are magnified images from upper panels.

Figure 2. High-grade HCMV-IE was frequently detected in infiltrated BC and DCIS but not in adjacent normal breast tissue. (A) Percentages of patients with HCMV-IE and HCMV-LA expression in infiltrated BC, DCIS, and adjacent non-tumor breast tissue. *n* is number of patients. Significantly higher number of patients with BC were positive for HCMV-LA in their tumor compared to those with DCIS ($p= 0.01$) (B) Percentages of patients with each grade of HCMV-IE and HCMV-LA expression in infiltrated BC, DCIS, and adjacent normal breast tissue. *n* is number of patients. In fifty one percent of the patients with infiltrating BC, >75% of the cells within the tumor expressed HCMV-IE protein compare with 22% in DCIS ($p= 0.002$) and 5% in adjacent non tumor tissue specimens ($p <0.0001$) (C) Percentages of patients with high-grade and low-grade expression of HCMV-IE and HCMV-LA. *n* is number of patients. High grade HCMV-IE (grade 3 or 4) was detected in 77% of patients with infiltrated BC compared to 39% in DCIS (grade 3 or 4) ($p= 0.004$) and 7% adjacent non tumor tissue specimens (grade 3 or 4) ($P <0.0001$).

Figure 3. Association between high-grade HCMV-IE expression and low levels of ER-α expression in BC. (A) High-grade HCMV-IE was significantly associated with low levels of ER-α expression in BC ($p = 0.02$). *n* is number of patients. 1+2+, low-grade HCMV-IE expression; 3+4+, high-grade HCMV-IE expression. (B) Representative photomicrographs of

BC show that ER-α expression was decreased/absent predominantly in tumors cells expressing HCMV-IE in the nucleus (a, b).

Lower panel present magnified images from upper panel.

Figure 4. Association between high-grade HCMV-IE and low levels of PgR expression in BC. (A) High-grade HCMV-IE expression correlated significantly with low levels of PgR expression (*p = 0.003*). 1+2+, low-grade HCMV-IE expression; 3+4+, high-grade HCMV-IE expression. (B) Representative photomicrographs showing high and low grades of HCMV-IE expression and PgR expression. Lower panel present magnified images from upper panel.

Figure 5. No significant association was found between high-grade HCMV-IE expression and absence of HER2 expression. (A) A trend was found between HER2-negative status and high-grade HCMV-IE expression in BC (p = 0.09). (B) Representative photomicrographs showing BCs that had high-grade or low-grade HCMV-IE expression and were HER2-positive or HER2-negative.

Supplemental Figure 1. No significant association was found between HCMV-IE expression and (A) Ki-67, (B) nodal status, (C) T stadium and (D) menopausal status in BC patients except for (E) histology grades (*p=0.03*).