Exploring the Prevalence of Ten Polyomaviruses and Two Herpes Viruses in Breast Cancer

Annika Antonsson1*, Seweryn Bialasiewicz2*, Rebecca J. Rockett2, Kevin Jacob2, Ian C. Bennett3, Theo P. Sloots2

1 Queensland Institute of Medical Research, Department of Population Health, Herston, Brisbane, Queensland, Australia, 2 Queensland Paediatric Infectious Diseases Laboratory, Queensland Children's Medical Research Institute, Sir Albert Sakzewski Virus Research Centre, Queensland Children's Health Service and The University of Queensland, Brisbane, Queensland, Australia, 3 Department of Surgery, Breast & Endocrine Unit, Princess Alexandra Hospital, Woolloongabba, Brisbane, Queensland, Australia

Abstract

Several different viruses have been proposed to play a role in breast carcinogenesis. The aim of this study was to investigate the prevalence of a subset of viruses in breast cancer tissue. We investigated the prevalence of 12 DNA viruses: EBV and CMV from the Herpesviridae family and SV40, BKV, JCV, MCV, WUV, KIV, LPV, HPyV6, HPyV7, and TSV from the Polyomaviridae family in 54 fresh frozen breast tumour specimens. Relevant clinical data and basic lifestyle data were available for all patients. The tissue samples were DNA extracted and real-time PCR assays were used for viral detection. The highest prevalence, 18% (5/54), was found for EBV. MCV, HPyV6, and HPyV7 were detected in single patient samples (2% each), while WUV, KIV, JCV, BKV, LPV, SV40, TSV and CMV were not detected in the 54 breast cancer specimens analysed here. Further investigations are needed to elucidate the potential role of viruses, and particularly EBV, in breast carcinogenesis.

Citation: Antonsson A, Bialasiewicz S, Rockett RJ, Jacob K, Bennett IC, et al. (2012) Exploring the Prevalence of Ten Polyomaviruses and Two Herpes Viruses in Breast Cancer. PLoS ONE 7(8): e39842. doi:10.1371/journal.pone.0039842

Editor: Christopher B. Buck, National Cancer Institute, United States of America

Received January 3, 2012; Accepted May 27, 2012; Published August 15, 2012

Copyright: © 2012 Antonsson et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This study was funded by the QCIMRI Program Grant 5006 (Queensland Children's Medical Research Institute). http://www.qcimri.org.au/funding/Programs.aspx. Annika Antonsson is supported by a fellowship from the Garnett Passe and Rodney Williams Memorial Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: annika.antonsson@qimr.edu.au

† These authors contributed equally to this work.

Introduction

Breast cancer is the most common type of cancer in women worldwide [1]. Only a small proportion of breast cancer cases, approximately 5%, are caused by hereditary mutations (such as BRCA1 and 2) [2], with the majority of breast cancers being sporadic or acquired in nature. Whilst there are some recognised risk factors for sporadic breast cancer such as late first full-term pregnancy, nulliparity, obesity and alcohol, only a small proportion of breast cancer cases actually display these risk factors. Hence, the aetiology of sporadic breast cancer remains enigmatic.

Infectious agents have been estimated to be responsible for 18% of human cancers [3], and a role of viral infection in breast carcinogenesis has been proposed. A number of studies have examined the role of different viruses with oncogenic potential such as human papillomavirus (HPV), mouse mammary tumour virus (MMTV), simian virus 40 (SV40), cytomegalovirus (CMV) and Epstein–Bar virus (EBV) in breast cancer tissue [4,5,6]. However, even though these viruses have been identified in human cancers [3], and a role of viral infection in breast carcinogenesis has not been thoroughly evaluated. In 2007, WU and KI polyomaviruses (WUV and KIV, respectively) were nearly simultaneously discovered by separate groups in the respiratory tracts of children presenting with respiratory disease [8,9]. A year later, Merkel Cell Polyomavirus (MCV) was discovered integrated into Merkel cell carcinoma (MCC) tissue, a rare but aggressive form of skin cancer [10]. Subsequent studies have confirmed the association between MCV, viral integration and MCC (up to 80% tumour positivity rate), and to this day, MCV presents the strongest evidence for polyomavirus involvement in human oncogenesis [11,12]. More recently, three additional polyomaviruses have been discovered; HPyV6 and HPyV7 on the skin of healthy human volunteers [13] and Trichodysplasia Spinulosa Polyomavirus (TSV) from the hair follicles of a heart transplant patient suffering from TSV’s namesake disease [14]. Like SV40, Lymphotrophic Polyomavirus (LPV) is a monkey-origin virus [15], however in the last few years, there have been several reports of its detection in the blood of immunocompromised patients [16,17]. The new members of the human polyomavirus family appear to have a tissue tropism preference for various forms of epithelial tissues, which also form the majority of breast cancer types [18].

In this study we investigated the prevalence of two members of the Herpesviridae family (EBV and CMV) together with ten members of the Polyomaviridae family (SV40, BKV, JCV, MCV, CMV, HPyV6, HPyV7, WUV, KIV, LPV).
WUV, KIV, LPV, HPyV6, HPyV7 and TSV) in breast tumour specimen.

Materials and Methods

Study population and samples

We analysed 54 breast cancer tissue samples that were removed as a part of treatment from patients undergoing surgery at the Wesley Hospital, Brisbane, Queensland, Australia between 2003 and 2007. These samples were used in a previously published study into the role of human papillomaviruses in breast cancer [6]. All tissue samples were confirmed by histology and the patients’ median age was 57 years, with a range of 31–88 years. We also had adjacent tumour free breast tissue from 10 of these women that served as controls. The median age for these 10 women was 55. Data on diagnosis, location, invasive grade, histological type, menopausal status, family history of breast cancer, cervical cancer diagnosis and HER2, oestrogen and progesterone receptors was also captured in an Access database.

Written informed consent was obtained from all patients, and the taking of frozen breast cancer tissue samples for this project was approved by the Princess Alexander Hospital Human Research Ethics Committee (PAH 2007/057).

Tumour tissue samples were snap frozen and stored at −80°C. Prior to extraction the samples were ground in 1.5 ml tubes on dry ice. The tissue samples were then homogenized in Trizol (Invitrogen, Carlsbad, CA), and DNA extraction was carried out following the protocol provided by the manufacturer. The DNA extracted from the breast tissue specimens was stored at −20°C until analysed.

Viral analysis

Viral detection was achieved with both published and specifically designed real-time PCR (rtPCR) assays. Samples were screened in duplex for JCV (J1) and BKV (V3a) using previously published methods [19,20]. Similarily, polyomaviruses TSV (VP1) assay [14], SV40 (SL1 assay) [20], as well as EBV [21], and CMV [22] were screened for in previously published singleplex assays. Based on available genomic sequences, rtPCR assays targeting MCV, LPV, HPyV6, HPyV7, WUV and KIV were designed and evaluated for cross-reaction with other viral species (Table 1). The LPV, HPyV6 and HPyV7 assays were run as simplex reactions, while the WUV and KIV assays were combined into a triplex.

Results and Discussion

Statistical analysis

For comparisons, we used the t-test for normally distributed continuous variables and chi-square or Fisher’s test (for small samples where the expected number in any cell was <5) for categorical variables. All analyses were conducted in SAS (version 9.2) and all significance tests were two sided at α = 0.05.

Results and Discussion

Fifty-four Australian breast cancer tumours were analysed for the presence of ten different viruses from the herpes and polyomavirus families.

The mean age of the 54 women studied was 57 years and the mean size of the study tumours was 24 mm (range: 8–120 mm), with a majority of patients diagnosed with invasive ductal

Table 1. Primer and probe sequences, in 5’ to 3’ orientation, of LPV, HPyV6, HPyV7, WUV and KIV assays used in this study, along with their genomic target.

| Oligo       | Sequence                     | Virus Target |
|-------------|------------------------------|--------------|
| LPV-VP2-F   | CATTGAAATAGAAGCAGTGATCTTG   | LPV VP2      |
| LPV-VP2-R   | AAACCTCCTATTCTGACGTTTG      |              |
| HPyV6-VP2-F | TTAGGAGCTGGACAAAGAATT       | HPyV6 VP2    |
| HPyV6-VP2-R | TCTGGGAGACTTTGGAATGTTG      |              |
| HPyV6-LT-F  | ACCAGTGGGTGATGAAAGACA       | HPyV6 LTAg   |
| HPyV6-LT-R  | CCGCTGAAATGGTTAAAAGGAAAGA  |              |
| HPyV7-LT-F  | ACCAGTGGGTGATGAAAGACA       | HPyV7 LTAg   |
| HPyV7-LT-R  | CCCCTCATAAGCATAAGTTGAATTTG  |              |
| HPyV7-LT-Pb | CAACTTTTCTGGGATATCTTGGTTGTC |              |
| HPyV7-VP2-F | GAGGAAGAACACTCCCAGATA        | HPyV7 VP2    |
| HPyV7-VP2-R | TTCACTCTATTCTGAGCTCTCAGAG   |              |
| HPyV7-VP2-Pb| ACTATACTCTATTGAGGTCTTTTGT   |              |
| WUV-F-Reg-F | GCCGACACGGCGTGATATA          | WUV NCCR     |
| WUV-F-Reg-R | TTCCGAGCCAGAAGAAT            |              |
| KIV-D-LT-F  | CACAGGTTGTTTATAAATTTTCTTATT | KIV LTAg     |
| KIV-D-LT-R  | GAATGCTACATACCCACGTTCATT    |              |
| KIV-D-LT-Pb | TGCATTGGCATCTGGATTTGACAGCA  |              |
| KIV-E-Reg-F | GAATCTCATCTCTTGGCACAGAGTTGA | KIV NCCR     |
| KIV-E-Reg-R | GGAATTAGAATTACAAGCTGGATTTGTC |              |
| MCV-LT1.1-F | AGCTCAAGAGATCCTCCATTGTTGTA  | MCV LTAg     |
| MCV-LT1.1-R | ACAATTGTCGCCAGAACA          |              |
| MCV-LT1.1-Pb| TTTCCAGAGGCTTCGTGGCATG     |              |

LTAg = Large T Antigen, NCCR = Non Coding Control Region.

doi:10.1371/journal.pone.0039842.t001
patients studied can be found in Table 2.

to be infiltrating lymphocytes, while no virus was detected in the recent paper, the EBV positive cells in breast tumours were found this age difference was not statistically significant (p = 0.09). In a EBV negative women (66 and 56 years old, respectively), however postmenopausal, and were on average 10 years older than the five EBV positive samples were grade 3 tumours, with the number of polyomavirus detections, we did not find any BKV positive compared to 2% in matched tumour-free breast tissue, SV40 in breast tumours found 22% of tumours to be SV40 polyomavirus SV40. A study by Hachana et al. [30] investigating DNA Viruses in Breast Cancer

| Table 2. Basic characteristic for study participants. |
|-----------------------------------------------|
| N | % |
|-----------------------------------------------|
| Total | 54 |
| Age (mean and SD) | 57.0 (11.9) |
| Menopausal status | Pre 13 24%, Peri 9 17%, Post 32 59% |
| Tumour size (mean and SD) | 24.3 (23.0) |
| Tumour size | >25 mm 41 76%, <25 mm 13 24% |
| Cancer type | Invasive Ductal 47 87%, mixed 1 2%, DCIS 1 2%, Invasive Lobular 5 9% |
| Grade | 1 10 19%, 2 23 42%, 3 20 37%, N/A 1 2% |
| Nodal Status | Positive 20 37%, Negative 34 63% |
| ER | Positive 43 80%, Negative 11 20% |
| PR | Positive 39 72%, Negative 15 28% |
| HER2 | Positive 8 15%, Negative 46 85% |
| Triple negative | Yes 6 11%, (ER, PR and HER2) No 48 89% |

doi:10.1371/journal.pone.0039842.t002

carcinoma (87%). Other relevant clinical features for the 54 patients studied can be found in Table 2.

The highest prevalence of the viruses investigated here in the breast tumour samples was 10% (5/54) which was found for EBV. None of the ten adjacent healthy tissues were EBV positive, including one originating from an EBV positive patient. Four of five EBV positive samples were grade 3 tumours, with the remaining one being grade 2. All five EBV positive women were postmenopausal, and were on average 10 years older than the EBV negative women (66 and 56 years old, respectively), however this age difference was not statistically significant (p = 0.09). In a recent paper, the EBV positive cells in breast tumours were found to be infiltrating lymphocytes, while no virus was detected in the malignant breast cells [24]. Due to insufficient original sample quantities, we were not able to perform \textit{in situ} hybridisation or immunohistochemistry on the EBV positive samples, and thus, could not determine the cellular origin of the detected virus.

MCV, HPyV6, and HPyV7 were detected in single cases (2% each) of the breast tumour samples. The MCV positive patient was a 58 year old woman with a grade 1 tumour of mixed type (ductal and lobular) breast cancer. The patient with the HPyV6 positive tumour was 52 years and had a grade 2 ductal breast tumour, while the HPyV7 positive patient was 78 years and had a grade 2 lobular breast tumour. The MCV, HPyV6 and HPyV7 positive samples produced high cycle threshold values of 31.9, 38.5, and 40.2, respectively, which is suggestive of low viral genome copy numbers. Given the evident low viral loads, and that MCV, HPyV6 and HPyV7 are polyomavirus with skin tropism, it is unclear whether these findings represent true infection or merely sample contamination originating from the skin during surgical excision or from skin shedding during sample processing.

WUV, KIV, JCV, BKV, LPV, SV40, TSV, and CMV were not detected in the 54 breast cancer specimens analysed in this study. JCV and BKV were discovered 40 years ago and findings of these two viruses have been reported in various types of tissues, but infections have mainly been associated with urinary tract diseases and mild upper respiratory tract infections [23]. Both viruses have been found to persist in kidneys, B-lymphocytes and the central nervous system (CNS) [26,27,28]. There are, to our knowledge, only one publication to date investigating JCV and BKV DNA in breast tumours [29]. This recent paper by Hachana et al. performed on Tunisian breast tumours did not find any BKV DNA positive tumours, but 23% of the specimens were found to be JCV positive. The JCV positive tumours in this paper were all invasive ductal type carcinomas. The majority of our samples (87%) were also of invasive ductal type, but we did not identify JCV in our population. Real-time PCR is generally accepted to be at least one log more sensitive than conventional PCR, and increasing PCR cycle numbers tends to increase the final yield and sensitivity of the assay. Considering Hachana et al used 35 cycles on their conventional PCR for the detection of JCV, while we used 45 cycles in a real-time PCR assay, we believe that assay sensitivity cannot explain the divergent JCV detections. In view of the Tunisian samples and our samples being similar breast tumour type (majority invasive ductal), and the previous study’s apparent lower JCV assay sensitivity, it is difficult to explain the vast difference in JCV detections between our sample sets. Furthermore, Hachana et al found a significant correlation between multiple viral infection (SV40, JCV and MMTV) and “triple negative” phenotype (ER, PR and HER2) [30]. Due to the low number of polyomavirus detections, we did not find any correlations with the “triple negative” phenotype and any other variables in our dataset.

None of our specimens were positive for the monkey origin polyomavirus SV40. A study by Hachana et al. [30] investigating SV40 in breast tumours found 22% of tumours to be SV40 positive compared to 2% in matched tumour-free breast tissue, which contrasts with the findings of our study. The previous study SV40 assay targeted the large T antigen gene, whereas we used a previously published assay targeting the structural VP2 gene. This choice of targets may have impacted our ability to detect any integrated SV40 genomes if their structural genes were truncated. Alternatively, the detection of SV40 DNA may have been due to endogenous contamination of reagents or samples by SV40 sequences, considering the ubiquity of SV40 sequence use in molecular biology.

CMV is an interesting virus in regard to breast cancer, even though there is no solid evidence of an involvement in breast carcinogenesis. Thirty to 40% of CMV infections are acquired during the first year of life and the route of transmission is mainly through breast milk from the mother [31]. Interestingly, one case-control study found an odds ratio (OR) of 4.0 for women seroconverting to CMV at least four years prior to being diagnosed with breast cancer compared to women who did not seroconvert [32]. When adjusting for parity and age at first birth, the OR for CMV seroconversion increased to 9.7 in the same study. However, CMV was not detected in our study population.

To conclude, we found very low or no prevalence to the viruses investigated in this study apart from EBV, although due to the histological limitations of the study, we could not draw robust
conclusions on EBV’s role in breast cancer. The study data suggests the lack of these DNA viruses’ involvement in breast cancer, however further investigations and larger studies are needed to elucidate if EBV plays a role in breast carcinogenesis.

Acknowledgments

We would like to thank Dr. Michael Pawlita (German Cancer Research Center (DKFZ), Heidelberg, Germany) for providing the LPV plasmid and Dr. Mariet Felkamp (Leiden University Medical Center, Leiden, The Netherlands) for providing the TSV plasmid.

Author Contributions

Conceived and designed the experiments: SB RJR KJ TPS. Performed the experiments: KJ RJR SB. Analyzed the data: AA SB. Contributed reagents/materials/analysis tools: ICB TPS. Wrote the paper: AA SB.

References

1. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, et al. (2010) Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 127: 2893–2917.
2. Campeau PM, Foulkes WD, Tischkowitz MD (2008) Hereditary breast cancer: new genetic developments, new therapeutic avenues. Hum Genet 124: 31–42.
3. Parkin DM (2006) The global health burden of infection-associated cancers in the year 2002. Int J Cancer 118: 3030–3044.
4. Amarante MK, Watanabe MA (2009) The possible involvement of virus in breast cancer. J Cancer Res Clin Oncol 135: 329–337.
5. Lawson JS, Heng B (2010) Viruses and Breast Cancer. Cancers 2: 752–772.
6. Antonsson A, Spurr TP, Chen AC, Francis GD, McMillan NA, et al. (2011) High prevalence of human papillomaviruses in fresh frozen breast cancer samples. J Med Virol 83: 2157–2163.
7. Lawson JS, Gunzburg WH, Whittaker NJ (2006) Viruses and human breast cancer. Future Microbiol 1: 33–51.
8. Allander T, Andreasson K, Gupta S, Bjerkner A, Bogdanovic G, et al. (2007) Identification of a third human polyomavirus. J Virol 81: 4130–4136.
9. Gaynor AM, Nisen MD, Whitley DM, Mackay IM, Lambert SB, et al. (2007) Identification of a novel polyomavirus from patients with acute respiratory tract infections. PLoS Pathog 3: e64.
10. Feng H, Shiula M, Chang Y, Moore PS (2008) Clonal integration of a polyomavirus in human Merkel cell carcinoma. Science 319: 1096–1100.
11. Fouloungue V, Dereeve O, Kluger N, Motes JP, Guillot B, et al. (2010) Merkel cell polyomavirus DNA detection in lesional and nonlesional skin from patients with Merkel cell carcinoma or other skin diseases. Br J Dermatol 162: 59–63.
12. Sautre-Guara X, Peter M, Avril MF, Laude H, Couturier J, et al. (2009) Merkel cell carcinoma of the skin: pathological and molecular evidence for a causative role of MCV in oncogenesis. J Pathol 218: 48–56.
13. Schowalter RM, Pastrana DV, Pumphrey KA, Moyer AL, Buck CB (2010) Merkel cell polyomavirus and two previously unknown polyomaviruses are chronically shed from human skin. Cell Host Microbe 7: 509–515.
14. van der Meijden E, Janssens RW, Lauber C, Bouwes Bavinck JN, Gorbalenya AE, et al. (2010) Discovery of a new human polyomavirus associated with trichodysplasia spinulosa in an immunocompromised patient. PLoS Pathog 6: e1000124.
15. zur Hausen H, Gissmann L (1979) Lymphotropic papovaviruses isolated from African green monkey and human cells. Med Microbiol Immunol 167: 137–153.
16. Delbov S, Tremolada S, Branchetti E, Elia F, Gualdo E, et al. (2008) First identification and molecular characterization of lymphotropic polyomavirus in peripheral blood from patients with leukoencephalopathies. J Clin Microbiol 46: 2461–2462.
17. Delbov S, Tremolada S, Elia F, Carloni C, Amico S, et al. (2010) Lymphotropic polyomavirus is detected in peripheral blood from immunocompromised and healthy subjects. J Clin Virol 47: 156–160.
18. Weigelt B, Reis-Filho JS (2009) Histological and molecular types of breast cancer: is there a unifying taxonomy? Nat Rev Clin Oncol 6: 718–730.
19. Hirsch HH, Mohaupt M, Klimkait T (2003) Prospective monitoring of BK virus load after discontinuing sirolimus treatment in a renal transplant patient with BK virus nephropathy. J Infect Dis 184: 1494–1495; author reply 1495–1496.
20. Pal A, Sirota I, Maudru T, Peden K, Lewis AM Jr (2006) Real-time, quantitative PCR assays for the detection of virus-specific DNA in samples with mixed populations of polyomaviruses. J Virol Methods 135: 32–42.
21. Kimura H, Morita M, Yabuta Y, Kanushima K, Kato K, et al. (1999) Quantitative analysis of Epstein-Barr virus load using by a real-time PCR assay. J Clin Microbiol 37: 132–136.
22. Watzinger F, Suda M, Peurunen S, Baumgartinger R, Ehner K, et al. (2004) Real-time quantitative PCR assays for detection and monitoring of pathogenic human viruses in immunosuppressed pediatric patients. J Clin Microbiol 42: 5189–5190.
23. Bialasiewicz S, Lombard SB, Whitley DM, Nisen MD, Slots TP (2009) Merkel cell polyomavirus DNA in respiratory specimens from children and adults. Emerg Infect Dis 15: 492–494.
24. Khan G, Philip PS, Al Ashari M, Houcimat Y, Daoud S (2011) Localization of Epstein-Barr virus to infiltrating lymphocytes in breast carcinomas and not malignant cells. Exp Mol Pathol 91: 466–470.
25. Dorries K (1998) Molecular biology and pathogenesis of human polyomavirus infections. Dev Biol Stand 94: 71–79.
26. Boldorini R, Brusato M, Veggiani C, Barco D, Andorno S, et al. (2005) Periodic assessment of urine and serum by cytology and molecular biology as a diagnostic tool for BK virus nephropathy in renal transplant patients. Acta Cytol 49: 235–243.
27. Dorries K, Vogel E, Gunther S, Czab S (1994) Infection of human polyomaviruses JC and BK in peripheral blood leukocytes from immunocompetent individuals. Virology 198: 59–70.
28. Ferrante P, Caddarelli-Stefano R, Omodeo-Zorini E, Vago L, Boldorini R, et al. (1995) PCR detection of JC virus DNA in brain tissue from patients with and without progressive multifocal leukoencephalopathy. J Med Virol 47: 219–225.
29. Hachama M, Amara K, Ziadi S, Gacem RB, Korbi S, et al. (2011) Investigation of human JC and BK polyomaviruses in breast carcinomas. Breast Cancer Res Treat.
30. Hachama M, Trimeche M, Ziadi S, Amara K, Korbi S (2009) Evidence for a role of the Simian Virus 40 in human breast carcinomas. Breast Cancer Res Treat 113: 43–50.
31. Onorato IM, Morens DM, Martone WJ, Santsfield SK (1985) Epidemiology of cytomegaloviral infections: recommendations for prevention and control. Rev Infect Dis 7: 479–497.
32. Cox B, Richardson A, Graham P, Gislefoss RE, Jellum E, et al. (2010) Breast cancer, cytomegalovirus and Epstein-Barr virus: a nested case-control study. Br J Cancer 102: 1665–1669.