Morphological and molecular analysis of a breast cancer cluster at the ABC Studio in Toowong

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Abstract

Introduction: In 2006 a possible cancer cluster was identified at the Australian Broadcasting Corporation (ABC) studio in Toowong, Brisbane. Thirteen women had been diagnosed with breast cancer which was estimated to represent a 6-fold increased rate over the general population. We undertook a molecular and pathological review of the cases and matched controls.

Methods: Slides were available for 12 ABC cases and 31 controls matched on age and year of diagnosis. Due to the limited amount of material available, only 6-8 cases and 26-29 controls were used for immunohistochemistry (ER, PR, HER2, CK5/6, SMA, p63, HPV and SV40), *in situ* hybridization (EBV), Southern blot analysis (MMTV) and gene expression profiling with the Illumina DASL system.

Results: No statistically significant differences were found between the cases and controls with respect to clinicopathological variables, presence of viral particles or gene expression.

Conclusion: The breast cancers from the ABC cluster appear to be similar to the range of breast cancers seen in the general population based on pathological review, immunohistochemistry sub-typing, presence of various viruses and gene expression profiling. Based on the current evidence from this small study, we did not find any molecular or pathological evidence that the ABC cases form a unique cohort.

Introduction

Cancer clusters involving small numbers of patients are relatively common, but only a few have been investigated.

Closer inspection of these clusters has found that for some clusters there is no significant increase in the risk of developing cancer (Mulla et al 2005; Roberts et al 2003). For cancer clusters which are significant in only a minority of these investigations is a causative agent found. A case study of angiosarcoma of the liver and brain cancer at a polymer production plant found an association with angiosarcoma and vinyl chloride exposure, but no association with brain cancer (Lewis et al 2003). Two studies have shown an increased risk in breast cancer in office workers exposed to strong magnetic fields (Milham et al 2004; Milham et al 1996). The analysis of a childhood leukemia cluster in Nevada (Rubin et al 2007), a breast cancer cluster among hospital nurses in Hungary (Tompai et al 1999) and a leukaemia cluster in Illawarra (Westley-Wise et al 1999) were unable to definitively show a common etiology. For many of the clusters, although a common etiology was not proven it could not be discounted, highlighting the difficulty in definitively associating a causal agent to a cancer cluster (Stewart et al 2010; Thun et al 2004). Nevertheless, in 2004 when staff at the Australian Broadcasting Corporation (ABC) premises in Toowong, Queensland, raised concerns about the number of breast cancer cases occurring among female employees at that site, investigations were initiated. In 2005, two studies attempted to evaluate the likelihood of the cancer cluster and to identify whether personal breast cancer risk factors or workplace exposure to electromagnetic fields were possible causal agents, but no firm conclusions could be drawn [7]. When an additional patient was diagnosed with breast cancer in 2006, an independent panel was constituted for a more systematic inquiry. That review included 550 women employed full- or part-time during the period from 1st January 1994 - 30th June 2006. A total of 13 women were diagnosed with breast cancer, with a possible additional case, and 10 of these were diagnosed while still working at the Toowong studio [8]. The expected number based on the Queensland rate of breast cancer was 1.6 cases [7]. The likelihood of this occurring by chance was estimated at approximately one in a million and this represented a greater than 6-fold increased risk compared to the general female population [8]. No specific cause of the cluster was identified, despite measurements of electromagnetic fields, ionizing radiation, and chemical contamination of water and soil, as well as traditional breast cancer risk factors [8].

In an attempt to elucidate the nature of this cluster, we instigated a review of the biology of the ABC patients' breast tumours. Breast cancer is a heterogeneous disease composed of several molecular pathology subtypes which may arise via different biological pathways. The working hypothesis was that a comparison to molecular subtypes may show that the tumours within the cancer cluster would have more similar morphological and/or molecular characteristics than those seen among tumours from a comparison group, and that this might reflect a common exposure to an unknown factor which might, in the long term, help to identify a novel breast cancer risk factor. This study also included an analysis of viral agents as there are some data to suggest a role for human endogenous retroviruses (HERVs) in breast cancer (Zur Hausen et al 2009).

Patients and Methods

Fourteen patients from the ABC breast cancer cluster were contacted and consent was obtained to study their tumours. All available samples were in the form of formalin-fixed paraffin-embedded (FFPE) blocks and histologic slides. For each ABC case, up

to three controls, matched for year of diagnosis and age of diagnosis (+/- one year), were retrieved from a large database at the Princess Alexandra Hospital. Ethics approval for the project was obtained from the Princess Alexandra Hospital Human Research Ethics Committee, with access to patient records approved by the Director General of Queensland Health under legislative requirements. Slides were de-identified and reviewed independently by two pathologists (SRL, GF). A proforma was developed to aid systematic reporting and the primary tumour characteristics were recorded for each sample. No slides were available for two ABC cases, no FFPE blocks were available for two cases and no material, even a pathology report, was available for one case (Table 1). Of the remaining 12 cases, one case had ductal carcinoma *in situ* (DCIS) only, and one tumour was too small for additional studies. Eight ABC cases had material suitable for inclusion in the tissue microarray (TMA). Twenty-nine control tumours were also included with two excluded (one was DCIS and another was too small for inclusion) (Table 1).

TMAs were constructed in duplicate from the case and control FFPE blocks. 4 μ m sections were cut from the TMA and immunohistochemistry was performed using standard protocols for ER (Roche Ventana clone SP1), PR (Roche Ventana clone 1E2), HER2 (Roche Ventana 4B5), CK 5/6 (Dako D5/16 B4), SMA (Dako 1A4), p63 (Biocare BC4A4), HPV (Biocare polyclonal) and SV40 (Calbiochem PAb 416). *In situ* hybridization was performed for HER2 (Ventana Inform®, Roche Ventana Medical Systems) and Epstein Barr virus (EBV) (EBV Early RNA Ventana Inform®, Roche Ventana Medical Systems). TMA results were available on six cases with no tumour or loss of the cores in two cases. A combination of TMA results and whole section results were used for clinicopathological classification.

Mouse Mammary Tumour Virus Analysis

Sufficient material was available for analysis of seven ABC cases and 18 control samples. Tumours were identified in hematoxylin- and eosin-stained specimens and comparable areas were removed from unstained slides for extraction of DNA and Southern blotting according to techniques previously reported [9, 10]. Southern blots were hybridized with a radioactive internal sequence of the envelope gene, and only those products of appropriate size with positive hybridization on two replicates specimens were considered positive or negative.

Clinicopathological data were analysed using R software. For the categorical groups, a two-sided Fisher's exact test was performed on the contingency tables with the null hypothesis that proportions are the same for ABC cases and controls.

Molecular Analysis

The sections were cut on the day of RNA extraction and macrodissected with a needle to enrich for areas with >50% tumour cells. RNA was extracted from 2-10 sections/sample. Cells were lysed in 260 μ l lysis buffer for 1hr at 70°C, proteinase K added and cells were incubated for a further 1hr at 55°C. 500 μ l of Tri reagent and 200 μ l chloroform was added to each sample; after mixing and centrifugation the upper phase was transferred to a fresh tube before isopropanol precipitation. RNA was pelleted and washed twice with 70% ethanol, then dried and resuspended in water. DNA was removed from the RNA by digesting samples with DNA-free (Ambion).

The Illumina Whole Genome-DASL (cDNA-mediated Annealing, Selection, Extension, and Ligation) assay which measures approximately 24,000 transcripts was performed as per manufacturer's instructions (Illumina.com). After hybridisation to the array the signal intensity for each transcript was measured using the Illumina Beadarray reader and the data extracted with Genomestudio (Illumina). The data were quality controlled using Genomestudio (Illumina) and the lumi bioconductor package [11]. Failed samples (n=3) were removed from analysis due to low average signal intensity (<100). Data was normalised with quantile normalisation then filtered using an Illumina detection score of >0.95 in at least two samples, which yielded 23693/24526 probes that were used in all further analyses. Unsupervised Hierarchical Clustering whereby transcripts which had a >2 fold change versus the mean were clustered. Pair wise analysis to determine differential expression was performed using the linear model and empirical Bayes method with Benjamini and Hochberg multiple testing correction [12]. Distance Weighted Discrimination (DWD) [13] coupled with Single Sample Predictor [14] was used to determine which sporadic molecular breast tumour subtype each sample resembled. The five centroid training set of 249 breast tumour samples from literature [14] was used in SSP to classify the tumours.

Results

No statistically significant differences were identified between the ABC case and control groups for any of the clinicopathological variables assessed (Table 2). The majority of the case and control tumours were ductal carcinoma of no special type. Tumour grade and size were similar between the case and control groups, as was the distribution of molecular subtypes as predicted by immunohistochemistry (IHC) [15, 16]. No cases or controls showed staining with HPV or EBV *in situ* hybridization nor were they positive with IHC for SV40 or HPV. MMTV was identified in 66.7%% of case samples and 59.3% of control samples but this difference was not statistically significant (p=1).

RNA was extracted from eight cases and 26 controls, and for three of these tumours (two cases and one control) two independent RNA extractions were performed from different areas of the tumour. Where possible the WG-DASL assay was performed in duplicate for each RNA sample. However, for three samples (one case and two controls) insufficient RNA was available and so these samples were assayed once only. An additional three samples (one case and two controls) failed quality control and were removed from analysis. In total, 68 samples were analysed successfully: eight cases (four in duplicate, two in quadruplicate, two singly) and 25 controls (22 in duplicate, one in quadruplicate and two singly). We found only 13 genes had a significant difference in expression between cases and controls (p=<0.05 with Benjamini and Hochberg multiple testing correction). The gene expression profiles of all 68 samples were clustered using unsupervised hierarchical clustering (Figure 1). The proportion of molecular subtypes present among the cases and controls was determined (Table 3). The classification was discordant for 6/29 duplicate pairs, but for three of these six samples the classification was either luminal A or luminal B. We assigned the final classification using the sample with the strongest prediction. There were no significant differences in the frequency of specific subtypes in cases vs controls. 50% of the cases (4/8) were classified as luminal A compared to 36% (9/25) controls. There were no luminal B or basal-like tumours within the small set of ABC

cases on gene expression profiling. Table 4 shows the relationship between the gene expression and immunohistochemistry subtypes.

Discussion

A clinicopathological review of the breast cancers showed no significant differences between tumour samples from the ABC cases and matched controls. The molecular subtyping showed that there were fewer luminal B and basal subtypes in the cases than controls, which does not support the hypothesis that *BRCAl*-carriers (which usually have the basal subtype) could account for the cluster [8]. Viral agents were investigated as possible aetiological factors for the ABC cluster because there are some data to suggest a role for human endogenous retroviruses (HERVs) in breast cancer [17]. However, there were no statistically significant differences in the prevalence of any virus between the ABC case and control groups.

There are no differences between the ABC cases and control tumours at the molecular level. Gene expression profiling is able to stratify tumours based upon phenotypic characteristics, but expression of only a few genes differed between the ABC cases and the controls, and unsupervised hierarchical clustering did not separate the tumours from the cases and controls. A supervised cluster analysis of the intrinsic genes that differentiate the breast tumour subtypes also showed that the ABC cases and controls did not cluster separately (data not shown). Together these findings indicate that the ABC cases and control tumours do not differ at a molecular level, and show that the ABC tumours are similar to the previously characterised sporadic molecular subtypes.

A high level of exposure to an exogenous agent in the environment might account for the cluster, but the environmental analyses performed previously found no evidence for such agents [8] [18] [19]. In 2009, a report was released on the risk of breast cancer among female employees of the ABC studios throughout Australia, including 5969 women employed between January 1994 and December 2005 [20]. This study reconfirmed the excess rate of breast cancer among ABC employees in Queensland, but no excess rate was observed among staff in the rest of Australia. A recent editorial [6] reviewed an updated analysis of the ABC cluster [21] which reported a much higher probability of the initial cluster than the one in a million originally reported, and concluded that the absence of an increased risk of breast cancer at other ABC sites in Australia, together with a failure to identify any causative agent and a revised probability of 1 in 25, means that the situation may have arisen by chance. Our results showing no shared pattern of morphological or molecular characteristics are consistent with such a conclusion.

In summary, the breast cancers from the ABC cluster appear to be morphologically and molecularly similar to the range of breast cancers seen in the general population based on pathological review, immunohistochemistry sub-typing, presence of various viruses and gene expression profiling. The limitations of our analyses of the tissue samples include the size of the tumours and the availability only of FFPE tissue; no frozen material or blood samples were available for the cases from the time of diagnosis. With the development of new technology, such as next generation sequencing, the possibility exists for a more in-depth analysis of the samples in the future. However, at this point in time, it seems reasonable to conclude that the cancers among the women of the ABC cluster are not unique. This may provide

reassurance to the affected women and their families that their work was not responsible for causing their breast cancers and reduce fear among others in the Australian broadcasting industry.

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Table 1: Summary of samples

	Slides available for review	FFPE material available	Included in TMA	RNA extracted
Case	12	12	8	8
Control	31	31	29	26
Total	43	43	37	34

Table 2: Clinicopathological characteristics of ABC case and control groups

	ABC Case Group		Control Group		p-value
Characteristic	n	%	n	%	
Age at Diagnosis					
50 years or less	11	79	26	84	
> 50 years	3	21	5	16	
Mean Age	42.79		44.35		
Tumour type					
Ductal NOS	11	79%	26	84%	
Ductal/Lobular	0	0%	1	3%	
Mucinous	1	7%	1	3%	
Micropapillary	0	0%	2	6%	
Unknown	1	7%	0	0%	
DCIS	1	7%	1	3%	
Histological Grade					0.34
GI	2	14%	8	26%	
GII	5	36%	10	32%	
GIII	4	29%	12	39%	
DCIS	1	7%	1	3%	
Unknown	2	14%	0	0%	
Tumour Size					0.09
<10mm	3	21%	4	13%	
10-20mm	7	50%	12	39%	
>20-50mm	1	7%	11	35%	
>50mm	1	7%	3	10%	
Unknown	1	7%	0	0%	
DCIS	1	7%	1	3%	
Hormone receptor status					1.0
ER+PR+	10	71%	19	61%	
ER+PR-	0	0%	1	3%	
ER-PR+	0	0%	0	0%	
ER-PR-	1	7%	10	32%	
Unknown	2	14%	0	0%	
DCIS	1	7%	1	3%	
IHC subtype					0.10
Luminal A	7	50%	19	61%	
Luminal B	3	21%	1	3%	
HER2	1	7%	5	16%	
Triple Negative	0	0%	5	16%	
DCIS only	1	7%	1	3%	
Unknown	2	14%	0	0%	
Lymph node status					0.78
Positive	6	43%	14	45%	
Isolated tumour cells	0	0	1	3%	
Negative	6	43%	15*	48%	

Unknown	2*	14%	1	3%	
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*One was DCIS only

Table 3. Proportion of molecular subtypes within the ABC cases and controls determined by gene expression profiling

	Basal-like	HER2	Luminal A	Luminal B	Normal-like	TOTAL
Control	8 (32%)	1 (4%)	9 (36%)	4 (16%)	3 (12%)	25 (100%)
Case	0	1 (12.5%)	4 (50%)	0	3 (37.5%)	8 (100%)

Table 4. Overall correlation between gene expression profiling and IHC subtypes.

	IHC subtype			
Gene expression Subtype	Triple Negative	HER2	Luminal A	Luminal B
Basal-like	5	2	1	0
HER2	0	1	0	1
Luminal A	0	0	13	0
Luminal B	0	0	4	0
Normal-like	0	1	3	2

Figure 1. Unsupervised clustering of all 68 samples successfully arrayed from 8 cases and 25 controls. Genes which varied 2-fold from the mean in at least 2/68 samples were clustered. Replicate samples are coloured similarly and cluster together; quadruplicate samples are marked with a bar and an asterisk. Cases are shown in blue, and controls in red.