

Osteogenic Role of Vascular Endothelial Growth Factor in Bone Regeneration

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Introduction

Many *in vitro* studies have shown that VEGF was able to stimulate osteogenesis apart from angiogenesis.¹ Deckers et al¹ suggested that vascular endothelial growth factor (VEGF) play an important role in the regulation of bone remodeling by attracting endothelial cells and osteoclasts and by stimulating osteoblast differentiation. The relationship between angiogenesis and osteogenesis was investigated by Rabie² and found to be closely related.

Currently, very few *in vivo* studies on the osteogenic capability of VEGF were done. Street et al³ used the rabbit model to apply VEGF locally using a pump to the site of bone damage and found that VEGF does promote new bone formation. They concluded that it might be an effective therapy to promote human bone repair.

Recently, orthopaedic tissue engineering strategies focused on the use of scaffolds as a delivery system for growth factors.⁴ Poly- α -caprolactone (PCL) is one of the synthetic polymers used as scaffold for bone regeneration which is non-toxic, biocompatible and highly compatible with osteoblast. It has a slow degradation rate which is ideal for load bearing applications in bone regeneration. It can be made into three-dimensional porous scaffolds and such a patent has been filed.⁵

This study was designed to investigate the osteogenic capability of VEGF in bone defects using fibrin glue as carrier and the fused deposition modelling (FDM) porous PCL as scaffold.

Materials and Methods

Thirty-two male adult New Zealand White rabbits (3.5- 4kg) were used in this study.

The surgical procedure involved an anteroposterior midline skin incision over the cranial vault of about 4 to 5 cm. Defects of 10 mm diameter were created in the parietal skulls of the rabbits using a trephine bur.

A total of 40 defects were created and randomly divided into 4 treatment groups. Twelve defects in 12 rabbits were grafted with PCL impregnated with rhVEGF (Calbiochem) and fibrin glue (Tisseel®, Immuno AG) as VEGF/ Fibrin/ PCL groups. Another 12 defects in 12 rabbits were grafted with PCL impregnated with fibrin glue only as Fibrin/ PCL groups (active control). Sixteen defects in 8 rabbits were used for the last 2 groups of control. Eight defects were grafted with PCL (active control) and the other 8 defects were left empty (passive control). All wounds were closed with interrupted 3/0 black silk sutures.

The rabbits were sacrificed and the tissue specimens were harvested at 1 week and 2 weeks (equal numbers from each group) for histological analysis. The specimens were fixed in formaldehyde, decalcified in 20% ethylene-diamine tetraacetic acid pH 7.2 (EDTA) and embedded in paraffin wax. Four sections of 5 microns were randomly selected from each specimen, sectioned and stained in haematoxylin and eosin. The amount of new bone formed was quantitatively and qualitatively analysed using the Olympus Micro Image software.

Ultra structural identification of cells involved in the healing of the experimental group, VEGF/ Fibrin/ PCL at 2 weeks, was also carried out. Tissues were harvested, fixed in glutaraldehyde, demineralised with 20% EDTA, postfixed in osmium tetroxide (OsO₄) and trimmed into 1 mm blocks and embedded in resin. The cells are identified using transmission electron microscope (TEM).

Data were analysed with statistical analysis package Statistical Package for Social Science (SPSS) Version 11.0. A preliminary two-way analysis of variance (Two-way ANOVA) was performed. Post hoc comparisons between groups were performed using Tukey HSD test. Student's t- test was used to compare the 1 week and 2 weeks groups. Level of statistical significance was set at $p < 0.05$.

Results

Quantitative

The amount of new bone formed for all groups is shown in Figure 1. Significantly more new bone was formed in both the groups grafted with VEGF/Fibrin/PCL compared to the control groups ($p < 0.01$). The amount of bone was found to be 352% ($p < 0.01$) higher in the 2 weeks VEGF/ Fibrin/ PCL group compared to the corresponding 1 week group. The amount of bone formed in the fibrin/PCL group was significantly more than that formed in the PCL and empty groups ($p < 0.01$).

Qualitative

Histologically, osteoid could be seen surrounded by osteoblasts at the frontal region of the newly regenerated bone, with osteocytes embedded in them (Fig. 2). Blood vessels were present in abundance in the surrounding area.

Mesenchymal cells, preosteoblasts, osteoblasts and osteocytes were identified under TEM. New bone formation was observed next to host bone. Numerous small blood vessels were seen in close proximity with the new bone formed.

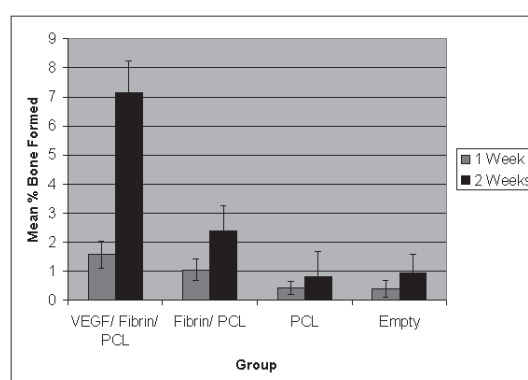


Fig. 1. Graph showing mean percentage bone formed.

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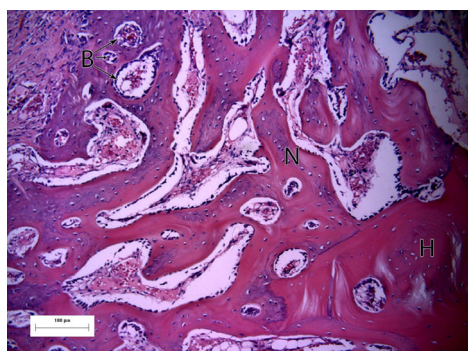


Fig. 2. VEGF/Fibrin/PCL at 2 weeks showing new bone (N), host bone (H) and blood vessels (B).

Discussion

Our results demonstrated that VEGF enhances early osteogenesis *in vivo* which concurred with the findings of Street et al.³ This is largely due to the angiogenic property of VEGF which is probably one of the most critical important factors for successful osteogenesis.^{2,3} As shown by histological analysis, numerous blood vessels were detected at the site of the new bone formation. In this study, as only a single dose of VEGF was applied locally to the site of defect, its effect was expected to be short-lived. Therefore, this study was designed to show the short-term effect of VEGF delivered by fibrin in PCL scaffold. For future studies into the long-term effect of VEGF, incorporation of the growth factor into the PCL scaffold would be the most ideal, as the release of VEGF will be sustained longer as PCL degrades slowly.

The ultrastructural identification of cells involved in the healing of the cranial bone defects revealed a normal healing pattern with a route of entry for preosteoblasts and secretory osteoblasts surrounding the new osteoid bone situated nearby to the host bone. This demonstrated active osteogenesis during early stage of bone regeneration.

Fibrin was used in this study to improve the handling of the VEGF, as VEGF is reconstituted in solution form. Fibrin may have slight osteogenic properties by itself and can provide an excellent fibrogenic network to enhance osteoblasts mobility when associated with a growth factor.⁶ Its biocompatibility makes it a good healing medium. In our study, fibrin glue could support the osteogenic activity of VEGF as shown by significantly more bone formed in the Tissue/PCL group compared to the PCL and empty groups.

PCL is a porous scaffold which serves as a three-dimensional template for initial cell attachment and subsequent bone tissue

formation. It is appropriate in this study as it serves as a resilient material for delivery of VEGF for bone regeneration. The PCL scaffold used in this study was fabricated in the Laboratory of Biomedical Engineering, BIOMAT, National University of Singapore (in collaboration with Temasek Polytechnic) by the FDM scaffold fabrication process. The FDM process forms three-dimensional objects from a CAD file as well as digital data produced by an imaging source such as computer tomography (CT) or magnetic resonance imaging (MRI). The process draws the designed model (scaffold) one layer at a time. This allows scaffolds of different micro-architectures (for example, porosity, wall thickness) to be custom made to requirements.

This study suggests that VEGF delivered by fibrin glue in PCL scaffold may be useful to promote osteogenesis in bone repair. This gives rise to many potential clinical applications. In orthodontics, these applications may include jaw reconstruction and repair of alveolar bone defects caused by trauma, pathology, clefts, early loss of teeth, periodontal disease or even loss of buccal plate of bone during extraction of buccally placed teeth.

Conclusion

VEGF delivered by fibrin glue in PCL scaffold does promote osteogenesis in early stage of bone regeneration and may be used for its osteogenic role in bone repair/ reconstruction application.

Acknowledgement

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Evaluation of Hemispheric Language Dominance in Adult Craniopagus Twins with Functional Magnetic Resonance Imaging (fMRI)

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Introduction

Conjoined Siamese twins are uncommon, with an estimated incidence of between 1:50,000 to 1:80,000 births. Of these, cranially conjoined or craniopagus twins are even rarer, with an incidence of 1 in 2.5 million births.¹⁻³ The evaluation of craniopagus twins, particularly in the adult setting, poses unique challenges to the attending surgeons and radiologists. Imaging to accurately define the anatomic fusion, the morphology of the underlying structures, including the vascular anatomy is important to guide therapy decisions, for surgical planning and prognostic information.

Apart from structural-morphologic evaluation, it is important to understand and define the underlying functional status of the brain pre-operatively. In particular, determination of the functionally important or eloquent brain areas controlling language function and the cerebral hemispheric language dominance is desired. Defining the eloquent language areas will help guide the operative approach should surgery be employed, and minimise morbidity. Such information may be obtained non-invasively by functional magnetic resonance imaging (fMRI) techniques using the blood oxygen level dependent (BOLD) effect. We present our experience in the fMRI evaluation of adult craniopagus twins to map the eloquent language areas during pre-surgical evaluation. To our knowledge, this is the first report in the medical literature of fMRI in craniopagus twins.

Materials and Methods

Patients

27-year-old female twins with parieto-occipito-temporal craniopagus were evaluated. Both had graduated from university, were intellectually normal and fluent in Persian (first language) and English. The right twin (A) was right-handed while the left twin (B) was left-handed. Structural-morphologic information had been obtained from computed tomography (CT), MRI and angiogram studies done in another centre in Singapore and previously in Germany.⁴ These indicated a partially shared cranium with the cerebral and cerebellar hemisphere components present in both twins, with their temporal and parietal lobes on the common fused side compressed and deformed. There was a common, shared superior sagittal sinus posteriorly.

fMRI Tasks

Four cognitive tasks were presented to each twin individually and in the same order consecutively. First was a visual perception task that required looking at a flashing checkerboard sequence. Significant fMRI BOLD activation was expected in the bilateral primary visual and visual association areas in this control task. Next was an English reading task in which each twin was required to silently read simple English words flashed one at each time. Such a task was expected to elicit activations at the major language areas of the brain, ie, Wernicke's area and Broca's area (or their homologous counterparts)

in addition to visual areas. The third task was an English picture-naming task, where each twin was required to silently name in English each object that appeared on the screen (adapted from Snodgrass & Vanderwart, 1980).⁵ Lastly, a Persian picture-naming task was presented, where each twin silently named in Persian the objects shown. These two tasks were expected to elicit bilateral prefrontal areas in addition to the areas mentioned for the other tasks. The computer-generated stimuli were presented via fiberoptic cables to binocular goggles that were adapted for each twin. Stimuli were designed and controlled by MacStim software.

Data Acquisition and Analysis

fMRI data was acquired on a 1.5T MRI scanner (NVi, GE Medical Systems, Wisconsin, MI) using a continuous T2*-weighted gradient-echo EPI sequence (per task 60 volumes with 8 axial slices each; TR: 2000 ms; TE: 40 ms; 8 mm slice thickness; FOV: 35 x 35 cm; 128 by 128 image matrix). Pre-processing and analysis of the data were done primarily using SPM99, based on the general linear model. Data were realigned, corrected for slice-timing and spatially smoothed with a Gaussian kernel of 14 mm FWHM. Data were modelled using a box-car function that was convolved with the haemodynamic response function (HRF) and a temporal derivative, which allows for the modelling of slight onset differences of around a second. Temporal smoothing of the data and design matrices was performed using a high-pass filter of 1/80 s and a low-pass filter using a Gaussian kernel of 4 mm FWHM. We reported activation if there were clusters of 3 or more voxels above a threshold of $p < 0.005$, uncorrected for multiple comparisons. Clusters significant at this level and which were activations expected due to apriori anatomical hypotheses were further evaluated statistically using a small volume correction.⁶ Results were independently verified using Brain Voyager v 4.9 software. The cerebral hemispheric dominance of task activation for each twin was calculated using the Laterality Index (L-R)/(L+R). Language dominance was defined as > 0.2 , as reported in other studies.⁷⁻¹⁰ Broad and homologous brain regions in each hemisphere, including regions around Wernicke's area and Broca's area, were defined.

Results

The visual perception task elicited significant BOLD activation in both the twins in the primary visual area. The visual association areas (precuneus, cuneus) were additionally activated in Twin A. The English reading task activated the language processing areas in the left cerebral hemisphere, namely the left middle temporal lobe around Wernicke's area and the inferior frontal cortex around Broca's area.

In Twin A, the picture-naming task in English activated the left inferior and middle frontal gyrus, bilateral superior and middle frontal areas, the left middle temporal areas, the left postero-inferior

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parietal cortex and visual areas. Twin B showed interesting activation in the left medial, superior temporal and the left inferior, middle temporal/occipital areas.

The Persian picture-naming task activated the left middle temporal area and the left superior frontal area in Twin A, while Twin B showed activation in the bilateral superior frontal and right middle temporal/occipital area.

Results were in accordance with normal data, but locating the activations was made more difficult by the complex neuroanatomy. Both twins were left hemisphere dominant for language function.

Conclusion

Non-invasive language mapping using fMRI has been demonstrated for the first time in total craniopagus. Conventional imaging equipment originally designed for single subjects was successfully adapted and tailored for the twins to permit adequate scan coverage of a larger region comprising 2 adult brains, while maintaining the diagnostic quality of the image data. Compared to our routine protocols, there was a relatively low signal-to-noise ratio (SNR) due to the scanning parameters, as we had to adapt towards a reasonable duration of scanning, to minimise head movement which will degrade image quality and affect data interpretation. Indeed, some head movement artifacts were encountered in the latter part of the fMRI scans for twin B. This diminished the ability to detect task-related BOLD responses and made it difficult to pinpoint activations due to the complex neuroanatomy. Nonetheless, as we performed a robust panel of fMRI tasks, there was sufficient data for analysis and conclusion. There was primarily left lateralisation of language function in both twins despite differing handedness.

fMRI is a useful non-invasive test for determination of eloquent brain areas controlling vital functions such as language, motor function and vision; and has been used routinely for pre-surgical planning and guidance in brain tumour surgery and epilepsy surgery. It offers a one-stop imaging solution whereby structural MRI scans are obtained together with functional data during the same scan session, and image fusion subsequently performed. Previously, determination of vital brain regions controlling various functions had to be performed with intra-operative electrode stimulation with the patient typically awake. For determination of language hemispheric

dominance, an invasive WADA test requiring the intra-arterial injection of sodium amytal during a catheter cerebral angiogram was previously routine. WADA testing has largely been supplanted by fMRI when the latter is available.

Our fMRI results for the twins are in accordance with the results of an independent WADA test performed at a separate examination (unpublished data – Lim SH et al, 2003). Indeed, the vast majority of normal subjects are left hemisphere dominant for language function, regardless of whether they are right or left handed.¹¹ Determination of the eloquent brain areas pre-operatively will help guide therapy decisions, selection of the best approach should surgery be contemplated and offer prognostic information.

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Best Oral Presentation Award Finalist – Surgery/Obstetrics & Gynaecology/Dentistry

BRCA1 c.2845insA is a Founder Mutation in Singaporean Malay Women with Early Onset Breast/Ovarian Cancer

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Introduction

Breast cancer is the most common cancer in Singaporean women. The incidence rate in 1993-1997 was 2.3 times higher than in 1968-1972, with an annual increase significantly higher in premenopausal

than postmenopausal women.¹ Of note, the Singaporean Malay ethnic group has shown the greatest increase in the incidence of breast cancer (4.4%) over the past 3 decades when compared to the Chinese and Indian subpopulations.

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Approximately 5% to 10% of all breast and ovarian cancer cases are believed to be attributable to the inheritance of germline mutations in high-penetrance, autosomal dominant susceptibility genes. The identification of one such gene, *BRCA1*,^{2,3} has rapidly led to the characterisation of mutations in this gene among high-risk families, as well as early-onset breast and/or ovarian cancer patients or tumour series in populations worldwide. Several founder and recurrent mutations have now been well documented in specific ethnic groups, such as the Ashkenazi Jews, and in Caucasian populations.⁴ Studies in Asian populations, though on the increase, have been few and amongst these Asian populations, recurrent and founder mutations have now been reported for the Japanese, Filipinos, Thais and Chinese.⁵⁻⁸

Given the increase in the incidence of breast cancer together with the rise in the number of patients presenting with early-onset breast cancer amongst the Singaporean Malays, we have characterised the germline *BRCA1* mutations in patients presenting with early-onset breast or ovarian cancer in order to evaluate the proportion of cancers attributable to mutations in this gene.

Materials and Methods

Study Patients

From 2001 to 2003, 72 consecutive unrelated Malay patients

TABLE I: *BRCA1* SEQUENCE ALTERATIONS IN SINGAPORE MALAY PATIENTS WITH EARLY ONSET BREAST/OVARIAN CANCER

Case	Exon	Sequence alteration	Amino acid change	Age (y)	Family history breast/ovarian cancer
<i>BRCA1</i> sequence variants					
Frameshift mutations					
N536 (Br)	11	c.2845insA	N909fsX5	35	Br33 (S)
N587 (Br)	11	c.2845insA	N909fsX5	44	Br60 (M), Br50 (MA)
N610 (Br)	11	c.2845insA	N909fsX5	35	Ov49 (M)
N633 (Br)	11	c.2845insA	N909fsX5	44	YTD
N521 (Ov)	11	c.2845insA	N909fsX5	49	Br37 (S), Br30 (MA), Br40 (MC)
N576 (Ov)	11	c.2845insA	N909fsX5	50	Nil
N590 (Ov)	11	c.2845insA	N909fsX5	40	Nil
Missense mutations					
N591 (Br)	6	c.335C>A	S72R	41	Nil
N536 (Br)	10	c.778C>A	S220Y	35	Br33 (S)
Neutral polymorphisms					
N33 (Br)	3	233G>A	K38K	38	Nil
N522 (Br)	3	233G>A	K38K	32	Nil
N599 (Br)	1	c.5106-68A>G	IVS16-68A>G	29	Nil
N616 (Br)	17	c.5106-68A>G	IVS16-68A>G	35	Nil
N599 (Br)	17	c.5106-68A>G	IVS16-92A>G	29	Nil
N616 (Br)	17	c.5106-68A>G	IVS16-92A>G	35	Nil
Intronic variants					
N591 (Br)	6	c.420+26CA>AC	IVS6+26CA>AC	41	Nil
N591 (Br)	6	c.432-44G>A	IVS5-44G>A	41	Nil
N511 (Br)	8	c.561-34T>C	IVS7-34T>C	34	Nil
N587 (Br)	18	c.5272+72G>C	IVS18+72G>C	44	Br60 (M), Br50 (MA)

ins: insertion; YTD: yet to be determined

Cancer site: Br = breast, Ov = ovary

Affected relatives: mother (M), maternal aunt (MA), maternal cousin (MC), sister (S)

TABLE II: GENOTYPES FOR THE *BRCA1* c.2845insA MUTATION IDENTIFIED IN 6 PATIENTS

Case	Intron 12 D17S1323	Intron 19 D17S1322	Intron 20 D17S855
N536 (Br)	155 159	119 127	142 148
N587 (Br)	157 159	115 127	142 142
N610 (Br)	153 159	119 127	142 146
N521 (Ov)	153 159	119 127	142 146
N576 (Ov)	157 159	121 127	142 144
N590 (Ov)	153 159	125 127	142 148

Common haplotypes are shown in **bold**

presenting to the National University Hospital were eligible for *BRCA1* testing if they had a diagnosis of breast or ovarian cancer before the age of 45 years and 50 years respectively. In addition, blood samples were collected from 114 unrelated normal subjects randomly selected from the general population. This control set was matched to the cases on the basis of race, gender and 5-year age intervals.

Molecular Studies

DNA isolation and mutation analysis of *BRCA1*

Total genomic DNA was extracted from the peripheral blood lymphocytes using standard procedures.⁹ The entire coding sequence, splice junctions and neighbouring intronic regions of *BRCA1* were screened using a combination of methods primarily single-strand conformation polymorphism (SSCP)¹⁰ and the protein truncation test (PTT).¹¹ All PCR products showing an electrophoretic variant pattern were directly sequenced. The deleterious mutations and genetic variants were named according to the practice of the BIC database,^{10,12} and any insertions or deletions were designated according to HUGO recommendations.¹³

Haplotype analysis

The disease-associated haplotypes in all the patients with the recurrent mutation and 76 controls were constructed by genotyping three intragenic *BRCA1* microsatellite markers D17S855, D17S1322 and D17S1323.^{14,15}

Results

In the 72 patients with early-onset breast or ovarian cancer under the ages of 45 years and 50 years respectively, 10 distinct *BRCA1* sequence alterations were identified in 14 patients.

The frameshift mutation, which produces a truncated protein signal at codon 914, was found in 7 apparently unrelated patients (Table I). Four of the 7 cases (57.1%) had at least a first or second-degree relative with breast or ovarian cancer. Three patients were early-onset breast cancer patients with positive family history of breast/ovarian cancer whilst the other 3 were ovarian cancer patients, of which only N521 reported a family history of breast carcinoma. The family history of patient N633 was yet to be determined at the time of publication. *BRCA1*c.2845insA was first reported in a retrospective study of apparently sporadic breast cancer patients¹⁰ and subsequently described in another study where it was found in 2 of 7 unrelated subjects of Malay ethnicity.¹⁶ None of the 114 unaffected controls were shown to harbour this mutation.

The genotypes at 3 *BRCA1* intragenic markers were examined for 6 unrelated affected patients with the mutation (Table II). No relatives were available to establish linkage phase, but for each of the loci, 1 allele was present in all 6 mutation carriers. For D17S1323 and D17S855, this allele was also common in the control individuals; but for D17S1322, this allele was only found twice in 152 chromosomes (1.3%). These data are consistent with all the c.2845insA mutations being found on a chromosome with a common haplotype, supporting an ancestral link. None of 76 unaffected controls shared the same haplotype as the 6 positive samples.

Two missense mutations of unknown significance, c.335C>A (S72R) and c.778C>A (S220Y), were detected.

Three neutral polymorphisms were detected in this study. Two patients harboured the single base substitution, c.233G>A; whereas the same 2 intronic polymorphisms, IVS16-68A>G and IVS16-92A>G, were found in 2 other breast cancer patients. These 3 polymorphisms have been reported previously in the BIC database and they are among the top 20 mutations frequencies that have been described by designation.

Discussion

Studies carried out in the Singapore population by our group and others have shown a similar prevalence of *BRCA1* mutations as has been seen in reports from western series.^{16,17} Interestingly, one common finding was that the same *BRCA1* deleterious mutation 2845insA was found in 2/7 Malay patients in one study suggesting the presence of a founder mutation.¹⁶ Follow-on studies confirming such a possibility, combined with the low incidence of mutations elsewhere in the gene, would have more significant clinical applications as a simplified mutation test limited to specific regions of the gene would allow for more widespread gene testing, and a more accurate estimate of mutation penetrance outside of multiple cancer families.

To test the above hypothesis, we have studied the spectrum of *BRCA1* mutations in 72 Singaporean Malay patients with early onset breast/ovarian cancer. The prevalence of *BRCA1* mutation in Southeast Asian breast and ovarian cancer patients, in particular of Malay ethnicity, has not been adequately reported and our study constitutes the largest cohort of such Malay patients to date. In this study, the prevalence of *BRCA1* mutation carriers in Malays was found to be similar to that of outbred populations and despite a full analysis of the entire coding region and adjacent intronic segments, all the deleterious mutations were found to be c.2845insA. In addition, none of the controls were found to carry this mutation. To the best of our knowledge, all reported *BRCA1* mutations in Malays have been shown to occur at this position with the exception of the c.5448insC mutation.¹⁸ Haplotype analysis of 6 out of the 7 patients with the recurrent c.2845insA mutation showed that shared haplotype was seen for all 3 intragenic polymorphic markers, strongly suggesting the presence of a founder effect at this locus. Interestingly, this haplotype was not seen in 76 controls which were analysed indicating that this may be a specific haplotype that exists within a specific subpopulation in the Malay population and that this haplotype is associated with increased susceptibility to breast/ovarian cancer.

Although *BRCA1* mutations have largely been reported to occur throughout the gene, recurrent and founder mutations have now been identified in specific subpopulations which have historically been geographically or politically isolated from surrounding populations. The present-day Singaporean Malays are part of a race of people inhabiting what is today Peninsula Malaysia and portions of adjacent islands of Southeast Asia, including the east coast of Sumatra, the coast of Borneo, and the smaller islands that lie between these areas, including Singapore. The Malays are typically characterised by their ethnically homogenous structure and low propensity towards immigration. In addition, the absence of the carrier haplotype among the 76 controls in our study also raises the possibility of a distinct sub-group at increased risk among the Malays.

These results have significant clinical applications as a directed search for the founder mutation is likely to account for the majority of mutation carriers in the population. Simplification of the mutation screening process will allow for a more widespread gene testing outside of hereditary breast and/or ovarian cancer syndromes, which

are rare in our general population. In addition, the identification of carriers outside of these families will also allow for a more accurate assessment of the penetrance of *BRCA* mutations, a vital requirement for genetic counselling and clinical decision-making regarding intervention, with either closer surveillance or prophylactic surgery.

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