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IDENTIFICATION OF *BRCA1* TUMOR SUPPRESSOR GENE EXPRESSION AS A DNA REPAIR GENE IN FROZEN CLEAVAGE EMBRYO CELL AT IIUM FERTILITY CENTRE

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Abstract

BRCA1 is known as the essential tumor suppressor gene in human that expresses a protein that called breast cancer type 1 susceptibility protein. It has two main functions which are repairing the damaged DNA and also inducing the apoptotic mechanism to eliminate the cell when mutated DNA could not be repaired. In this study, all the embryo samples that have been used were considered as grade 1 according to Embryo Grading. This study aimed to investigate *BRCA1* gene expression in relation to the human embryo quality. The RNA from the cleavage embryo was extracted and was tested by Reverse-Transcriptase Polymerase Chain Reaction (rt- PCR) and gel electrophoresis. The results showed that *BRCA1* gene expression was expressed in frozen cleavage embryos. Furthermore, the statistical analysis revealed that there are no significant differences among the fragmentation rates and also in Pre-embryo Genome Activation (EGA) and Embryo Genome Activation (EGA) phases. This study would be beneficial in terms of controlling the DNA repair gene activity during the pre-implantation phase. The expression of *BRCA1* could be a useful health assessment indicator for embryos.

INTRODUCTION

BRCA1 gene expression is an essential gene for DNA repair system in cleavage embryo. This study has been designed to investigate *BRCA1* gene expression in early embryo development. Poor quality cleavage embryos expressed higher *BRCA1* expression due to significant quantities of damaged and fragmented DNA in that embryo [1]. Recently, in Pre-implantation Genetic Diagnosis (PGD), *BRCA1* is one of the genes which has been diagnosed to detect later onset

of potential ovarian cancer and hereditary breast cancer. It also has been used to diagnose disorders, which have symptoms that decrease the quality of life rather than life expectancy.

The families that have a history of a severe genetic disorder used PGD to select the best quality embryos for *in vitro* fertilization (IVF). However, to use PGD for adult-onset conditions, it is a need to obtain ethical approval by ethical committees and also have relevant justification when there is no well-known intervention to overcome the serious conditions which significantly burdensome. The less serious or lower penetrance of conditions, PGD for adult-onset conditions is ethically acceptable as a matter of reproductive liberty. When the risks of PGD are higher than their benefits, it should be discouraged in order to preserve the quality of life. For example, *BRCA1* has an association with the potential development of breast cancer. The presence of *BRCA1* gene does not predict with certainty that an individual will ever develop breast cancer. This preliminary study provided some scientific information about *BRCA1* gene which could enhance the development of gene testing in PGD.

BRCA1 gene expression has DNA repair pathways that should be activated to produce an embryo that can succeed in implanting and initiating a clinically viable pregnancy at early stages should be highlighted. *BRCA1* gene has a significant role in controlling the DNA repair system in human cells. Furthermore, poor-quality mice embryo has indicated the existence of *BRCA1* mutation [2]. The mutation *BRCA1* caused genetic instability in human growth development. In severe cases, the mutation could lead to fatality. Therefore, this study aims to investigate *BRCA1* gene expression in relation to the human embryo quality.

MATERIALS AND METHODS

Embryo Sampling

The sample of embryos was collected from the patients that had visited the IIUM Fertility Centre with ethical approval from International Islamic University Malaysia Research Ethics Committee (IREC). Samples were selected to undergo identification of *BRCA1* expression by using conventional polymerase chain reaction (PCR) and agarose gel electrophoresis. Eight samples (Sample 1 (S1), Sample 2 (S2), Sample 3 (S3), Sample 4 (S4), Sample 5 (S5), Sample 6 (S6), Sample 7 (S7) and Sample 8 (S8)) of frozen embryo were taken out from liquid nitrogen storage and they were transferred into 1.5 mL Cryovial tube by using a syringe. Cancer cell lines (pooled sample which contained HeLa and breast cancer) was used to indicate the band of *BRCA1* being expressed for the positive control (tense band) whereas pooled of embryo samples were used to indicate the band of *BRCA1* being expressed as negative control to test the reliability of the primer (faint band). All embryos (S1-S8) that were used in this study exhibited all criteria listed in Table 1. Therefore, all samples have been classified as grade 1 in accordance with Table 2. The fragmentation of the embryo samples is the uneven division of the cells. The percentage of the fragmentation was illustrated in Table 3.

Table 1: Embryo scoring criteria

Criteria	Description
Criteria 1	Day 2: 2-4 cells (26-44 hours) Day 3: 6-8 cells (66-70 hours) Day 4: Compacted / morula (90-100 hours)
Criteria 2	< 10% fragmentation
Criteria 3	Proportion of blastomeres

Table 2: Cleavage scoring grades

Grades	Description
Grade 1	Embryo exhibits all criteria
Grade 2	Embryo exhibits 2 of 3 criteria
Grade 3	Embryo exhibits all 1 of 3 criteria
Grade 4	Embryo exhibits none of the criteria

Table 3: Embryo samples description

Samples	Cell No.	Fragmentation
S1	2- 4 cells	< 5% fragmentation
S2	3-4 cells	< 5% fragmentation
S3	4-5 cells	< 5% fragmentation
S4	2-3 cells	< 5% fragmentation
S5	5-7 cells	< 5% fragmentation
S6	6-7 cells	< 5% fragmentation
S7	4 cells	< 5% fragmentation
S8	2-5 cells	0% fragmentation

RNA extraction and first-strand cDNA synthesis

Total RNA was extracted from all embryo samples and cell line pooled samples using the PureLink® RNA Mini Kit following the manufacturer's instructions. The quality of extracted and amplified RNA was assessed by using the NanoDrop ND-1000 spectrophotometer. The purified RNA was converted into cDNA by using SuperScript® III First-Strand Synthesis System for rt-PCR Kit (Invitrogen, USA). The following procedure was designed to convert 1 pg–5 µg of total RNA or 1 pg–500 ng of poly(A)+ RNA into first-strand cDNA) following manufacturer's instruction. The product of cDNA was tested with NanoDrop ND-1000 spectrophotometer which then the cDNA yield was run by gel electrophoresis.

The RT-PCR, Detection and Semi-quantification of rt-PCR products

Reverse Transcriptase Polymerase Chain Reaction (rt-PCR) was performed with 2.0µL of total RNA in 25µL by using TopTaq DNA Polymerase kit (Qiagen) following manufacturer's instructions. PCR amplification of the cDNA product was carried out by adding 0.125 µL of TopTaq DNA polymerase for 35 cycles in a commercial thermocycler in the following conditions: 3 minutes initial denaturation at 94°C, 30 seconds denaturation at 94°C, annealing at 58.8°C for 30 seconds, extension for 1 minute at 72°C and final extension at 72°C for 10 minutes. Aliquots and 100 bp DNA ladder (Invitrogen, USA) were loaded in 2% agarose gels containing 0.1 µg/ml ethidium bromide and turned on the electric power. Photographs of the gels were generated and visualized by using an image analyzer (Alpha Innotech). The intensity of the bands corresponding to PCR products was determined using AlphaImager HP Imaging System (Alpha Innotech, USA). Meanwhile, the peak density of intensity of each band has been assessed by densitometry analysis performed by using the public domain NIH Image 1.63 program**.**

Primer Sequence

Breast cancer susceptibility gene, *BRCA1* primer sequence was taken from a previous study [3] and predesigned by Integrated DNA Technologies (IDT). Table 4 shows the primer sequence used for measuring the cleavage embryo cell gene expression with the accession number of the sequence used for primer design.

Table 4: Primer sequence used for measuring cleavage embryo cell gene expression and the with accession number.

Gene	Primer Sequences	NCBI accession no.
Breast cancer susceptibility gene, *BRCA1*	Forward primer: 5'-TGTGCTTTTCAGCTTGACACAGG-3' Reverse primer: 5'-CGTCTTTTGAGGTTGTATCCGCTG-3'	XM_006722041.1

Statistical Data Analysis

All the data were expressed using mean ± error bar with standard deviation. A non-parametric test was used due to the limited number of samples for each group. Median and interquartile range (IQR) were calculated. Kruskal Wallis was tested to identify any significant difference between the expressions of *BRCA1* in grade 1 embryos with the different embryo fragmentation groups (no fragmentation and 5-10% fragmentation) whereas Mann Whitney was used to identify a significant difference between the expression of *BRCA1* in grade 1 embryos with two different groups of Embryo Genome Activation (EGA). The result was considered non-significantly differences for $p \geq 0.05$.

RESULTS AND DISCUSSION

Figure 1 indicates the gels of *rt*-PCR amplification products of *BRCA1* (255 bp) for each sample of embryo. As represented by Figure 1, the bands of *BRCA1* were expressed in embryo samples of S1, S3, S4, S5, S7 and S8 while the *BRCA1* was not expressed in embryo samples of S2 and S6. Cancer cell lines (Pooled sample which contained HeLa and Breast Cancer) were used to indicate the band of *BRCA1* being expressed for the positive control (tense band) whereas a pooled sample of all embryo samples was used to indicate the band of *BRCA1* being expressed for negative control (faint band).

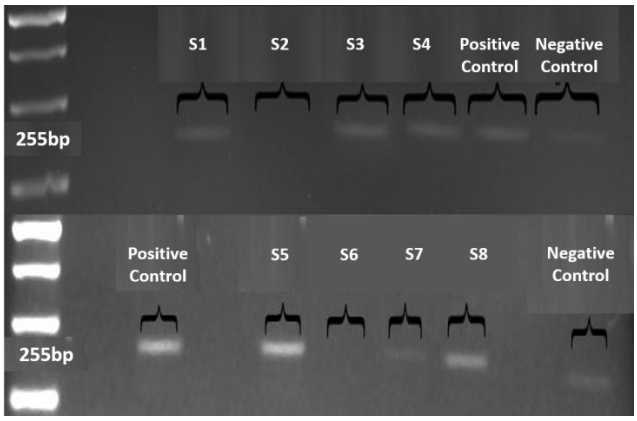

Figure 1: Representative gels of rt-PCR amplification products of *BRCA1* (255 bp) for all grade 1 samples (sample 1 (S1), sample 2 (S2), sample 3 (S3), sample 4 (S4), sample 5 (S5), sample 6 (S6), sample 7 (S7) and sample 8 (S8)). The 100 bp DNA ladder (Invitrogen, USA) marker is on the left of each gel. The band of *BRCA1* being expressed in embryo samples (S1, S3, S4, S5, S7 and S8) while the band of *BRCA1* being not expressed in embryo samples (S2 and S6). Cancer cell lines (pooled sample which contained HeLa and breast cancer) was used to indicate the band of *BRCA1* being expressed for the positive control (tense band) whereas pooled of embryo samples were used to indicate the band of *BRCA1* being expressed as negative control in order to test the reliability of the primer (faint band).

Figure 2 shows the peak density of *BRCA1* of each sample. The highest value of peak density of *BRCA1* could be seen in S5 which is 0.999 whereas; the lowest value of peak density of *BRCA1* could be seen in S7 which is 0.148. For samples S2 and S6, both of them have zero value of peak density of *BRCA1* because there are no bands detected on both samples in this study.

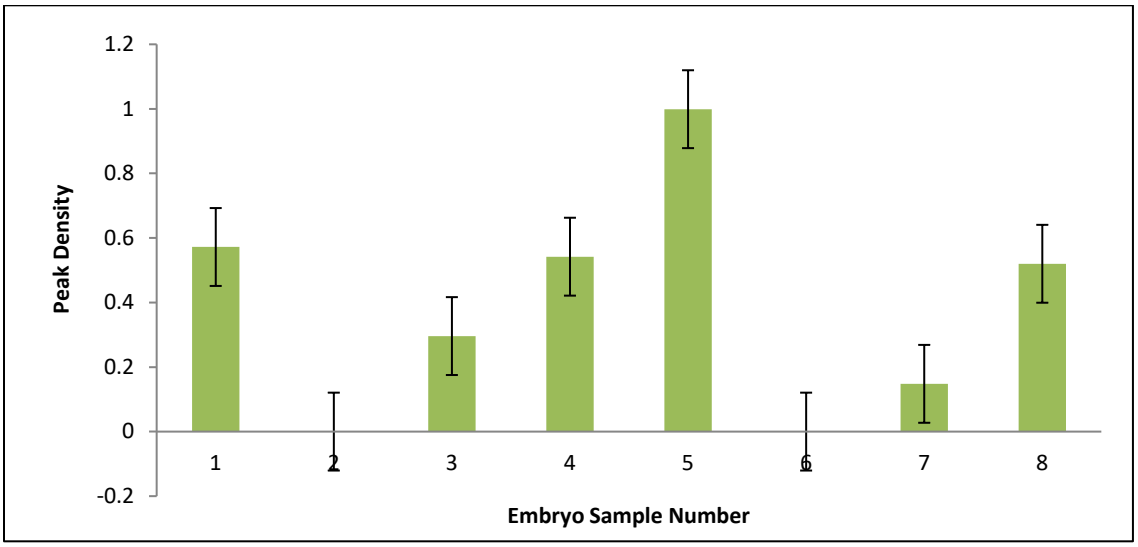

Figure 2: Peak density of *BRCA1* in each embryo samples

Figure 3 shows each value of peak density *BRCA1* value obtained from the non-fragmented embryo group and fragmented embryo group. The two-tail p-value from Mann-Whitney test is 0.314. The horizontal line represents the median expression (0.546 for non-fragmented group whereas 0.222 for fragmented group) and the box encompasses 50% of data points (first quartile to the third quartile). Maximum and minimum data points are represented by upper adjacent value and lower adjacent value (vertical lines). Thus, the peak density in the non-fragmented group is higher than the peak density in the fragmented group.

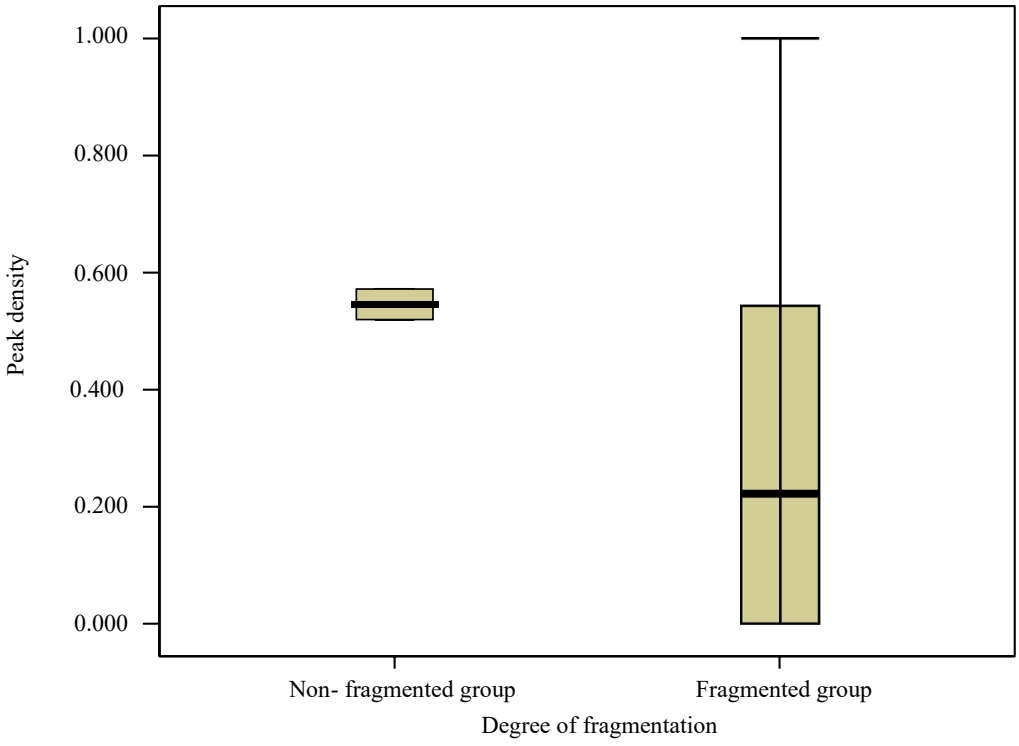

Figure 3: Peak density of *BRCA1* in grade 1 embryo that grouped into non-fragmented embryo group and fragmented embryo group, *p*-value >0.05

BRCA1 gene orchestrates virtually in every aspect of cellular life which started from growth followed by metabolism to cell division and differentiation especially in DNA repair mechanism [4]. DNA repair mechanism is essential during the cleavage embryo development to ensure embryo viability for IVF treatment [1]. Knowledge of this gene was revealed much information about the DNA repair processes in a cell. Sometimes, it could indicate the viability and health of embryo cells. The improvement of understanding of this gene activity in human pre-implantation embryos would have significant clinical value and great scientific findings about the information of the gene. This information has the potential to assist in specifically characterized the DNA repair mechanisms underlying key developmental of embryo, including compaction, cavitation, Embryonic Genome Activation (EGA) phases, and differentiation of the inner cell mass and trophectoderm. The analysis of this gene expression is providing the data which is concerned about embryo viability and metabolic requirements relevant to IVF treatment.

As reported earlier, reverse transcriptase-polymerase chain reaction (rt-PCR) was used to evaluate the expression of *BRCA1* gene [3,5]. In this study, *BRCA1* expressions were expressed in 6 embryo samples out of 8 embryo samples (Figure 1). *BRCA1* gene was previously found to accumulate at very high levels in fully grown 5-10 cells of embryo [1]. EGA is activated during 5-10 cells of the embryo [6]. At EGA phase, *BRCA1* gene was expressed within the cell to activate the DNA repair mechanism pathway [7]. It could be deduced that the higher expression of *BRCA1* could be due to the embryo cells have the damaged DNA or the DNA was damaged during the first mitotic division. On the other hand, the up-regulation of *BRCA1* has been related to the response to the DNA damage. For cleavage-stage embryos the first opportunity to upregulate transcription of this gene is after the embryonic genome becomes active, and consequently, embryos inheriting damaged DNA or chromosomes may have to wait until this stage before *BRCA1*- mediated repair can be activated. These findings suggest the importance of *BRCA1* in the embryonic genome activation phase during embryo maturation and pre-implantation embryo development. Approximately, the results of this study showed similarity with the results that have been reported by Wells et al.[8]. The expression of *BRCA1* gene has a relationship with fragmentation rates of embryo as well as with the influence of EGA [8]. However, by comparing three different fragmentation rates and also by comparing both Pre-EGA and EGA phases with peak density of *BRCA1*, due to p-values > 0.05, there are no significant differences were indicated for both comparisons.

The peak density of *BRCA1* gene in three different fragmentation rates was not significant with the *p*-value > 0.05 (Figure 3). However, it has been reported that the expression of the *BRCA1* gene has a relationship with fragmentation whereby *BRCA1* gene highly expressed in embryo which contained a little or no fragmentation [1]. Meanwhile, those that were highly fragmented displayed low expression of this gene. There is no significant difference (p-value > 0.05) between peak density of *BRCA1* with EGA groups. EGA groups in this study were divided into two different groups, which were the Pre-EGA group that contained 2-4 cell embryo while EGA group that contained 5-10 cell embryo. As asserted by, embryo which contained 5-10 cell embryo significantly expressed *BRCA1* gene whereas the expression of *BRCA1* in 2-3 cell embryo is vice versa [8]. However, the results showed there were no significant differences in variables that have been tested. These results contradicted the previous study by Wells et al. [1]. The assumption that could be suggested for obtaining these results is the use of a small size of embryo samples. Only 8 samples of embryo were collected, extracted and tested for this study. A small sample size is not enough to test statistical assumptions. The larger the sample size could provide more accurate and precise results. Thus, as shown by figure 2, one deduction in this study can be made which; there is no significant difference of peak density of *BRCA1* between S5 (the highest peak density) with S7 (the lowest peak density).

All embryo samples in this study have been classified as grade 1 embryos. There were many human pre-implantation embryos generated by using assisted reproductive techniques fail to form a viable pregnancy [1]. Only 25% of embryos successfully transferred during pre-implantation. From this statement, one assumption could be speculated, even though the selected embryo for IVF is a grade 1 embryo, it could not be granted to produce a viable pregnancy. Some external or internal factors could disturb the rate of potential pregnancy level. However, these factors have remained unknown. As supported by one study, any changes of expression of genes in embryos that are needed during embryo development were associated with alteration of embryo anomalies [8]. In *BRCA1* context, *BRCA1* is the center of the DNA repair mechanism that would interact with other proteins to repair the DNA damage [9]. Any abnormal changes of *BRCA1* gene expression during embryo development would cause genetic instability that subsequently could contribute to cancer development [10]**.** Moreover, the expression of *BRCA1* in an embryo has shown the existing of DNA damage in that embryo [1]. From the results of this study, *BRCA1* gene was expressed in the grade 1 embryo. Even though, in embryo grading, grade 1 embryos should be free from DNA damage. It could be deduced that there are existed of external or internal factors that are still unknown. The existing *BRCA1* expression in grade 1 embryo could be considered as one of the factors that caused non-viable pregnancy. Meanwhile, *BRCA1* was required in embryonic cellular proliferation in mice because *BRCA1* also could be considered as a positive growth regulator [2]. If this factor was taken into account for this study, all embryos should have *BRCA1* gene expression in early embryo development because it is essential for embryonic proliferation and growth. This statement could

answer the role of *BRCA1* gene that is expressed in grade 1 embryo.

RNA purity plays an important role in gene expression analysis. The compromised RNA integrity, purity and stability were suggested to lead to unreliable results in gene expression studies [11]. In this study, there were three samples do not reach RNA purity within the range; 1.9-2.1, which are S2, S3 and S6 due to the small cell numbers of each embryo**.** The results from this study, S2 and S6 did not expressed *BRCA1* gene while S3 expressed *BRCA1* gene with lower peak density. These results have shown to us the RNA purity has an impact on gene expression analysis as stated by Vermeulan et al [11].

In conclusion, this study highlighted a general pattern of *BRCA1* gene expression and also revealed the dynamic fluctuations in *BRCA1* gene activity that occur throughout pre-implantation development. The information could be utilized to generate gene expression profiles which highly concerning the characterization of characteristics of grade 1 embryo. These patterns of *BRCA1* gene expression in embryos in their developmental stage may have superior viability to those displaying atypical gene activities. This study also shown the possibility of semi-quantification of *BRCA1* gene in frozen cleavage embryo using rt-PCR could be done even though the numbers of cells in each sample too little. Thus, analysis of *BRCA1* gene that might serve as an indicator of embryo viability could be combined with an assessment of genes responsible for single-gene disorders for preimplantation genetic diagnosis (PGD). Alternatively, imprinted *BRCA1* gene could be assessed intensively to provide reassurance that no aberrant expression had been induced by assisted reproductive treatment.

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