Investigating the Relationship Between Cadherin Expression and Invasiveness in Breast Cancer

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Abstract
Cadherins are transmembrane receptors that allow cells to interact and are involved in epithelial-mesenchymal transition and metastasis of cancer. Cadherins can be classified into three types and when these cadherins are expressed incorrectly, it can lead to various types of cancer with varying degrees of invasiveness. The expression levels of the cadherins tested for were determined by extracting RNA from the cell and reverse transcribing the RNA into cDNA. The cDNA was then quantified for each cell line using real-time polymerase chain reaction (qPCR) and the data was normalized by calculating the ΔΔCt value. Cadherin 4 was shown to be expressed in only the MDA-MB-231 cell line while cadherin 3 was expressed in only the SKBR3 cell line. Cadherin 11 and cadherin 5 were not expressed in any of the cell lines tested. These results made it difficult to identify the relationship between invasiveness and cadherin expression, although if determined this could help to better diagnose and treat breast cancer.

Introduction

Cadherins represent a large superfamily of transmembrane receptors, which allows cells to interact with each other, and allows cell signaling. Cadherins are classified into three basic types, type I, type II and type III, and they are all expressed in mammary glands. Type I cadherins are also known as classical cadherins and they include P-cadherin and R-cadherin. Type II cadherins contain two tryptophan residues before the first calcium binding site, which causes the cadherin to make a different cytoplasmic connection than type I cadherins. Type II cadherins include cadherin 5 and cadherin 11. Type II cadherins are also called atypical cadherins because they have a GPI anchor rather than a transmembrane domain [1]. While the role of type I cadherins has been studied extensively, little is currently known about type II cadherins and their role in cancer [2].

The expression of cadherins has been shown to play an important role in breast cancer so it is important to understand the expressions of cadherins in order to better diagnose and treat cancer. Cadherin expression in breast cancer has been shown to influence cadherin switching and may also be important in controlling epithelial-mesenchymal transitions (EMT) and mesenchymal-epithelial transition (MET) [1]. Cadherin switching can occur when the type of cadherin expressed in epithelial cells switches to a cadherin expressed in mesenchymal cells, contributing to EMT [1]. During EMT there is a loss or redistribution of junction proteins and an increase in invasiveness and motility [3]. Cancer cells can then migrate to another location in the body and undergo MET in which the cells return to their epithelial phenotype [1].

The cadherins tested for in this study were cadherin 3, cadherin 4, cadherin 5 and cadherin 11. Cadherin 3 and cadherin 4 are both categorized as type I cadherins. Cadherin 3, also known as P-cadherin is overexpressed in breast cancer and may enhance the migration of breast cancer cells [4]. Not much is known about the function of cadherin 4, also known as R-cadherin, but in breast cancer its expression may be repressed to give cells a metastatic phenotype [1]. Cadherin 5 and cadherin 11 are type II cadherins [1]. Cadherin 5 expression is altered in invasive breast cancer, which enhances the ability of endothelial and mammary epithelial cells to adhere together [1]. Cadherin 11, or OB-cadherin, is also expressed in breast cancer, and has been shown to promote the migration of cancer to bone [5].

The aim of this study was to determine the relationship between invasiveness and cadherin expression in four human breast cancer cell lines, MDA-MB-231, SKBR3, T47D, and MCF7. Once the levels of expression was determined in each cell line, the relationship between cadherin expression and invasiveness of each cell line can be identified.

Materials and Methods

Cell Culture:

All cell lines were cultured and grown in DMEM-F12 plus 10% fetal bovine serum (FBS), 1% sodium pyruvate, and 1% PSG.

Extraction of RNA:

The cells were cultured in a 6 well plate and extracted using TRIZOL Reagent (Life Technologies) and placed in microcentrifuge tubes in -80 ° C overnight. Chloroform was added to each tube and the tubes were mixed by shaking and incubated at room temperature for 2-3 minutes. The tubes were then centrifuged for 15 minutes at 12,000xg at 4°C. The aqueous layer was then removed and placed into fresh tubes. Isopropyl alcohol was then added to the aqueous layer and incubated for 10 minutes at room temperature. The tubes were then centrifuged at 12,000xg for 10 minutes at 4°C. The supernatant was then removed and the pellet was washed with 75% ethanol. The samples were mixed by vortexing and centrifuging at 7500xg for 5 minutes at 4°C. The pellet was then air dried and dissolved in RNase free water. The samples were then incubated at

55-60°C. The yield and concentration of RNA was then quantified using the Biotek spectrophotometer.

RT-PCR:

RT-PCR was performed using 1.0 µg of RNA from each cell line and using the Verso cDNA kit. The samples were run in the Bio-Rad DNA Engine® Thermo Cycler using the following cycles: 1 cycle of 47°C for 60 minutes and then 95°C for 2 minutes.

qPCR:

The following primers were used for the MDA-MB-231, SKBR-3 and T47D cell lines: cadherin 3 upstream, 5'-CACCAACCATCATCCCGACA-3'; cadherin 3 downstream, 5'-TAGCCGCCTTCAGGTTCTCA-3', cadherin 4 upstream, 5'- ATTTACCGCCAGCACGTTTG-3'; cadherin 4 downstream 5'-GACCATGCCCTCGTTGGTTA-3'; cadherin 5 upstream, 5'-CCCTTCTTCACCCAGACCAA-3'; cadherin 5 downstream, 5'-TGAAAGCGTCCTGGTAGTCG-3'; cadherin 11 upstream, 5'-TCAAGGGCCCCAGAAATCAC-3'; cadherin 11 downstream, 5'-TTGAGCTCATCACGTCAGGG-3'. For MCF7 cells, the primers for cadherin 3 and cadherin 4 remained the same while the primers for cadherin 5 and cadherin 11 were changed to: cadherin 5 upstream, 5'-ATGACAATGCCCCGGAGTTT-3'; cadherin 5 downstream, 5'-TGTTGGCCGTGTTATCGTGA-3'; cadherin 11 upstream, 5'-CAAGCCACTTTCCAACCAGC-3'; cadherin 11 downstream, 5'-GCCTGCTGTGTTATCTCGGT-3'. The primers were diluted to a concentration of 1M and then combined with Platinum® SYBR Green Supermix in a ratio of 1:2. This mixture was then added to a 96 well plate (7.5 mL per well). The cDNA made from RT-PCR was diluted 1:20 and then 2.5 mL was added to each well of the 96 well plate. Deionized water was added to some wells instead of cDNA to be used as a control. The following cycling parameters were used for PCR: 39 cycles of 3 minutes at 95°C, 95°C for 10 seconds, 55°C for 10 seconds, and 72°C for 30 seconds, then 95°C for 10 seconds, and 1 degree intervals from 65°C to 95°C for 5 seconds each.

Gel Electrophoresis:

A 2% agarose gel was prepared. The samples were prepared by adding loading dye to the sample in a 1:10 ratio. Sample mixture was then added into each well and one well was loaded with DirectLoad Wide Range DNA Marker (Sigma Aldrich). The gel was then run at 100V for 30 to 40 minutes and then visualized using the FluorChem E System (Protein Simple).

Results

R-Cadherin mRNA is expressed in MDA-MB-231 cells in vitro.

MDA-MB-231 is classified as claudin-low and has a triple negative basal phenotype which means that the cell line lacks expression of ERα, PR, and HER2. [6]. One study classified this cell line as having level 4 invasiveness, which is the most invasive [2]. In the current study it was determined which of the four cadherins, cadherin 3, cadherin 4, cadherin 5, and cadherin 11, were expressed in this cell line. To investigate this, qPCR was performed on four different samples (passage 141, 142, 143, and 144). It was determined that MDA-MB-231 cells do not express cadherin 3, cadherin 5 and cadherin 11 (Table 1) as shown from the melting curve when compared to the no template control (NTC). Cadherin 4 (R-cadherin) however, showed increased expression when compared to the no template control. GAPDH was used as a control and was shown from qPCR to be expressed in all passages of this cell line (Figure 1 and Table 1).

P-Cadherin mRNA is expressed in SK-BR-3 cells in vitro.

Next the cadherins expressed in SKBR3 were determined. SKBR3 cells have a HER2 classification and are negative for ERα and PR but positive for HER2 expression [6]. One study classified this cell line as having an invasiveness of 2 on a scale of 0 to 4 [2]. To identify which cadherins were expressed in SKBR3 cells, qPCR was performed on three different samples (passage 5, 7, and, 8). The results showed that SKBR3 cells express cadherin 3 (P-cadherin) mRNA (Table 1) when compared to the no template control in the melting curve graph. The melting curves for cadherin 4, cadherin 5, and cadherin 11, when compared to the no template control, indicate that these genes are not expressed in this cell line since there was no difference in melting temperature of the products (data not shown). GAPDH was used as a control and was shown to be expressed in all samples of SKBR3 cells (data not shown). cDNA from passage 7 of the SKBR3 cell line originally indicated that cadherin 5 was also expressed, however when a second sample of RNA was used to make cDNA that sample did not show expression of cadherin 11 mRNA when compared to the control (data not shown). Since this was only seen in one sample of SKBR3 it is believed that cadherin 5 is not expressed in this cell line.

To further confirm that cadherin 3 is expressed in SKBR3 cells, a gel was prepared and electrophoresed using samples from the completed qPCR. Based on the primers used to amplify cadherin 3, the qPCR product should have had a length of 93 bp. When the gel was visualized, bands were present for passage 7 and 8 for cadherin 3 (Figure 2) but bands were not present at 93 bp for cadherin 4 because this cadherin was not expressed in SKBR3 (data not shown). The primers for cadherin 4 were unable to adhere to the cDNA since the mRNA for cadherin 4 isn't produced and therefore there was no amplification products seen on the gel after qPCR. This confirms that cadherin 3 is expressed in SKBR3 cells but cadherin 4 is not.

T47D and MCF7 cell lines lacked mRNA expression of any cadherins.

Lastly the cadherin expression in T47D cells and MCF7 cells was determined. The T47D and MCF7 cell lines both have a luminal A classification. These cell lines are positive for ERα expression, negative or positive for PR expression and negative for HER2 expression [6]. MCF7

and T47D cell lined are also both classified as having a level 2 invasiveness on a scale of 0 to 4 [2]. To examine the cadherins present in T47D and MCF7 cell lines, qPCR was performed on 3 different samples of each cell line. Passages 119, 120, and 122 were used for the T47D cell line and passages 23, 24, and 25 were used for the MCF7 cell line. The melting curve graphs created from qPCR showed no expression of cadherin 3, cadherin 4, cadherin 5, or cadherin 11 for either of the cell lines when compared to the no template control (Table 1). GAPDH was used as a control and was shown to be expressed in all samples of both cell lines indicating that cDNA was present and the test was working properly (Table 1).

Discussion

Attempting to determine the relationship between invasiveness and cadherin expression presented a challenge based on the results from qPCR of each cell line. Since none of the cells lines tested expressed the same cadherins, the relationship to aggressiveness was unable to be determined. However, since MDA-MB-231 is the most invasive cell line examined and was the only cell line to express cadherin 4, cadherin 4 expression may play a role in the invasiveness of this cell line. Based on the data collected, it is unclear whether these cell lines do not express the cadherins or if the test was not accurate in determining expression of each cadherin. One reason the test may not have been accurate is because the forward and reverse primers used for each cadherin were too complementary to each other. Evidence of this is seen on the electrophoresis gel at the 50 bp marker (Figure 2). For both cadherin 3 and cadherin 4, faint lines can be seen on the gel indicating that some of the primers may have annealed together rather than amplifying the cDNA that was expressed. In future research other primers should be tested and more gels can be analyzed to possibly confirm the presence of primer dimers. In future research a Western Blot should also be performed to confirm that cadherin 3, 4, 5 and 11 proteins are being expressed in the cell lines. If there is no protein expression then this would support the results seen with qPCR.

Cadherin 5 expression has been shown to have an effect on invasiveness in melanoma [7] and cadherin 11 has also been shown to be more highly expressed in invasive breast cancer cells [2]. These studies indicate that cadherins do have an effect on the invasiveness of cancer. This means that learning the relationship between invasiveness and expression of cadherins could help to identify the most invasive forms of breast cancer so that breast cancer can be better diagnosed and treated in the future. Before this can happen however, a cadherin that is expressed in all these cell lines must be identified. Future research should be focused on determining which cell lines express each cadherin so that the expression of those cadherins can be used to correlate invasiveness.

Conclusion:

The results show that MDA-MB-231 cells express cadherin 4 and SKBR3 cells express cadherin 3. T47D and MCF7 cell lines did not show expression of any cadherins. Future work needs to be done to confirm these results and determine the relationship between invasiveness and cadherin expression once a common cadherin is determined for all cell lines examined.

References

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Figures and Tables

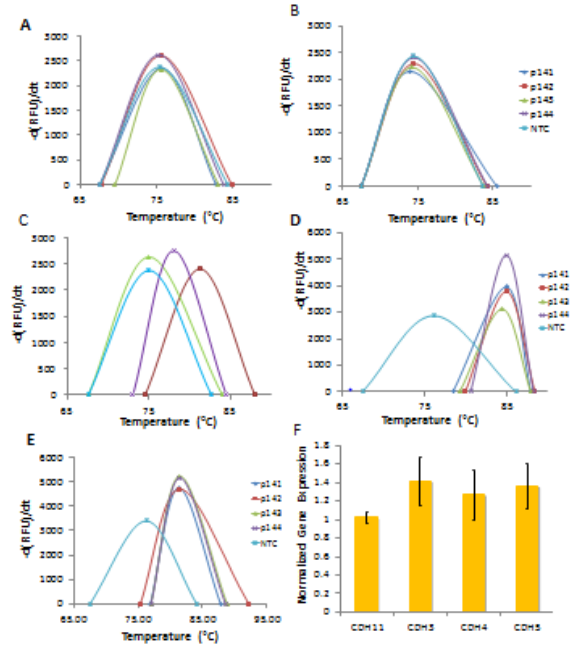

Figure 1. Results for cadherin gene analysis by quantitative real time polymerase chain reaction (qPCR) for MDA-MB-231 cell line**.** Representative melting curves for qPCR products for the primers directed against cadherin 5 (A), cadherin 11 (B), cadherin 3 (C), cadherin 4 (D), and GAPDH (E). The average ΔΔCt values were also calculated to determine the expression of each gene (F).

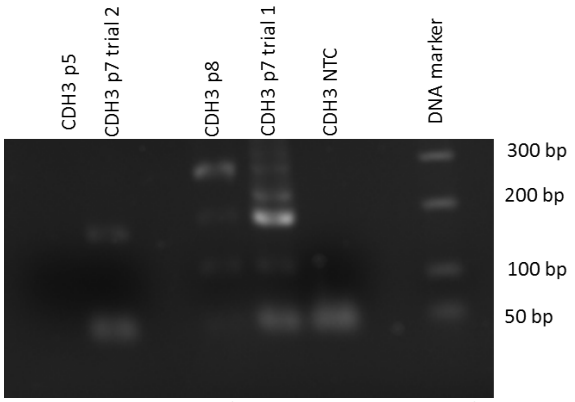

Figure 2. Photograph taken of the gel electrophoresis performed using the qPCR products for cahderin3 for the SKBR3 cell line.

Table 1. Overall results for the expression of each of the five genes in each of the four cell lines. GAPDH is used as a control. These results were determined from the qPCR results. Positive indicates that the gene is expressed in that cell line and negative is used to show that the gene is not expressed by that cell line.

	Genes Expressed				
Cell Line	**CDH 3**	**CDH 4**	**CDH 5**	**CDH 11**	**GAPDH**
MDA-MB-231	-	+	-	-	+
SKBR3	+	-	-	-	+
T47D	-	-	-	-	+
MCF7	-	-	-	-	+

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Biography

Alaina Kaiser is currently a senior and will graduate in May 2015 with a B.S. degree in both Forensic Science and Biochemistry. She is a Chemistry and Biology tutor and an Organic Chemistry laboratory teaching assistant. Alaina is originally from Rochester, NY and she hopes to pursue a Masters in cellular and molecular biology. She also plans on continuing this research over the next year.