Activation, Expression, and Deregulation of the Notch Signaling Pathway in Human Breast Cancer

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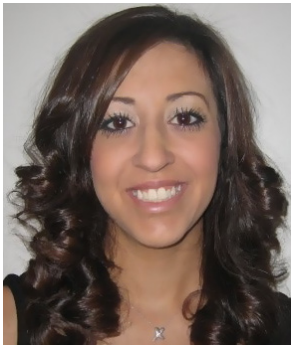

Writer's Comment: As a student in a University Writing Program course, I was given the opportunity to write a literature review of 6–10 primary research articles on a recent research topic. I hold a keen interest in the field of oncology, particularly in alterations that occur in cellular signaling pathways and their connection with cancer, and therefore chose this topic for my paper. My passion for the subject turned an ordinary assignment into an exciting opportunity to delve deeply into my topic. Having never written a literature review before, I learned how to rummage through countless research articles and obtain all pertinent information quite quickly. Because of the opportunities available here at UC Davis, I have been able to extend my knowledge beyond what is required by professors, and, in turn, climb my way up a ladder of success.

—Rachel Smith

Instructor's Comment: My first impressions upon reading Rachel Smith's paper on Notch signaling were that it was very technical and that Rachel really knew her subject well. She had been interested in doing research on breast cancer well before the UWP 102B class, and this literature review gave her the chance to focus on a particular area of the puzzle and find out what the most recent research had to say. I was very impressed with Rachel's dedication to this subject matter throughout the course, and it was no surprise to me that she produced such a strong literature review. She found eight articles to include, of which four had been published within a month before the assignment was due. Rachel processed these articles thoroughly and synthesized and organized their contents intelligently. While lay readers may have difficulty following the technical ins and outs of the Notch signaling pathway, Rachel's paper will help any reader understand the general principles behind the pathway and how its deregulation can lead to malignancies.

—Jared Haynes, University Writing Program

Introduction

Breast cancer is the leading diagnosed cancer among women today. With many aggressive subtypes and consequent deaths, understanding the mechanisms involved in the progression of this disease is crucial for proper treatment.

Deregulation of the Notch signaling pathway has been associated with many malignancies, including invasive breast carcinomas and tumorigenesis (Ma *et al.,* 2010; Harrison *et al.,* 2010). The Notch signaling pathway is intimately involved with cell growth, proliferation, differentiation, and apoptosis (Stylianou, Clarke, and Brennan, 2006). Four proteins that make up this pathway, Notch1 through 4, become activated when interacting with "Jagged" or "Delta-like" ligands on the cell's surface (Mittal *et al.*, 2009). Once activated, a Notch intracellular domain (NICD) is cleaved, released, and sent to the nucleus where it interacts with RBP-Jk/CBF-1, a transcriptional regulator (Mazzone *et al.,* 2010) of downstream proteins, including those of the Hes family (Stylianou, Clarke, and Brennan, 2006; Reedijk *et al.*, 2005). The accumulation of NICD and expression of Hes family proteins can be used as a determinant of Notch activation (Stylianou, Clarke, and Brennan, 2006).

Aberrant Notch activation has been shown to exist in certain cell lines of human breast cancers. Understanding the proteins involved in Notch's regulation, as well as experimenting with appropriate inhibitors to these proteins, will bring us closer to the successful treatment of those who suffer from this disease.

Expression of Notch Ligands in Breast Cancer

In order to determine which Notch receptors play a role in the development of breast cancer, the cancerous tissue must be evaluated for the expression of specific Notch ligands (Mittal *et al.*, 2009). Studies done by Stylianou, Clarke, and Brennan (2006) and Mittal *et al.* (2009) used reverse transcription polymerase chain reaction (RT-PCR) analysis of breast cancer tissue for Notch, Jagged, and Delta-like ligands (Stylianou, Clarke, and Brennan, 2006; Mittal *et al.*, 2009). Stylianou, Clarke, and Brennan (2006) found that Notch1 and Notch3, as well as Delta-like4, Jagged1 and Jagged2 ligands are expressed in the same vicinity, suggesting that these ligands cooperate with one another.. Mittal *et al.* (2009) concluded that Notch receptors are present in both normal and cancerous tissue, though the cancerous tissue samples showed a higher

concentration of Notch1, Notch2, and Jagged expression. The researchers failed to detect Delta-like and Notch3 expression in normal tissue samples but found a significant increase in these levels in cancerous tissues. Also, Notch4 levels showed no increase in expression even though these levels were the highest in both normal and cancerous tissues.

Reedijk *et al.* (2005) took a different approach in verifying the expression of Notch signaling proteins in cancerous tissues. They observed mRNA and ligand-specific antisense riboprobes by using photomicrographs of tumor samples hybridized *in situ*. They found all four Notch proteins to be highly expressed in the tumors, as well as a co-localization of Jagged1 (Jag1) and Notch3, suggesting an interaction between these two ligands.

Activation of Notch through NICD and Hes Expression

Because simple expression of Notch ligands does not constitute activation of the Notch signaling pathway, Stylianou, Clarke, and Brennan (2006) and Mittal *et al.* (2009) sought to identify the activation of downstream targets as well as the accumulation of NICD through antibodies specific for these proteins. Stylianou, Clarke, and Brennan (2006) showed accumulation of NICD and expression of Hey1, a Hes family protein, through Western blot analysis. In accordance with this data, Mittal *et al.* (2009) found that 75 percent of all observed cases showed buildup of NICD and representative staining for Hes family proteins. The expression of up-regulated downstream targets and accretion of NICD levels indicate active Notch signaling present in cancerous breast tissue samples.

Controls of Notch Regulation

Because NICD presence signifies Notch activation, an overexpression of NICD can signify increased Notch activation. Mazzone *et al* (2010) sought to determine the product of infecting a cancer cell line, *in situ,* with high levels of NICD. Only 10–20% of these NICD-infected cells began to proliferate at high levels, while the majority of the immortalized cells failed to colonize, showing that NICD can affect tumor growth. Certain proteins may use levels of NICD as an indicator to downregulate the signaling pathway, therefore slowing Notch activation and its tumor-initiating effects.

Along with altering the downregulation of Notch comes knowledge of its upregulators. Ma *et al.* (2010) sought to determine the effects on the Notch signaling pathway resulting from application of mTOR, a serine/threonine protein kinase that incorporates signals from cellular pathways to regulate cell growth and proliferation. After treating mouse embryonic fibroblasts with overexpressed mTOR, scientists used blot analysis to show significantly elevated levels of the Hes1 protein. Because this protein signifies Notch activation, it was practical to conclude that mTOR is a positive regulator of Notch signaling.

The Loss of Numb Expression and its Effects on BCSC

The deregulation of Notch signaling was presumed to be due to a malfunction of a Notch inhibitor, Numb (Mittal *et al.*, 2009). An absence of Numb expression was observed after testing for the presence of NICD and Hes expression (Mittal *et al.*, 2009). Furthermore, immunohistochemical analysis of normal breast tissues showed a profusion of Numb expression.

These results were confirmed through use of similar methods on breast cancer tissues. Mittal *et al.* (2009) observed tumors that showed positive and negative mixed expression of the Numb marker and found that positively expressed tumors showed Notch activation through cleaved Notch1 and Hes family proteins. Consequently, the Notch and Hes proteins may have developed a resistance to Numb mediation in some cancers (Mittal *et al.*, 2009). While Notch signaling in normal tissues is regulated by Numb, cancerous tissues either have lost expression of this inhibitor or contain proteins with a developed resistance to Numb mediation.

Notch Inhibitors Reduce BCSC Activity and Tumor Size

Insight into negative effects on Notch regulation proposed by loss of Numb expression has raised suspicions about the benefits Notch inhibitors may have for cancerous tissues. In a study testing the effects of Numb on BCSC (breast cancer stem cell) degradation, all but 10 percent of the cells were eliminated after stimulation and incubation with a Numb-activated peptide (Mine *et al.,* 2009). After testing the response of BCSC to many effectors, scientists showed the highest rate of CSC reduction with the Numb-activated peptide.

Harrison et al. (2010) applied Notch inhibitors, DAPT and DBZ, in vitro to BCSC to determine the effect of treatment on stem cell activity. This treatment not only reduced the expression of a downstream target, Hes proteins, but also that of the Notch4 and Notch1 proteins. However, no reduction in the size of mammospheres was observed. Alternatively, Ma et al. (2010) noted a reduction in the proliferation of tumorigenic cells after application of a Notch inhibitor, Notch-negative, both in nude mice and human cancer cells. By utilizing upstream regulators and Notch inhibitors in Notch signaling, scientists have developed a possible therapy for BCSC activity and tumor reduction.

Ras Signaling and Notch1 Cooperation

The Ras signaling pathway is known to assist in activation of the Notch signaling pathway through G-coupled proteins and receptor tyrosine kinases (Eckert *et al.*, 2004). Mutation or deregulation of the Ras pathway can lead to aberrant Notch signaling (Eckert *et al.*, 2004). Eckert *et al.* (2004) sought to determine by Western blot analysis of Ras-GTP in cultured tissue samples whether there is an up-regulation of unmutated Ras in breast cancer cell lines. Elevated Ras-GTP levels were found in six of the ten cell lines under study, suggesting a possible explanation for the increase in Notch signaling that has been observed in breast carcinomas.

To understand the direct effects of up-regulated Ras signaling, the specific Notch proteins affected by Ras signaling must be known. Weijzen *et al.* (2002) investigated this relationship through obstruction of post-translational modification of the Ras pathway through a farnesyltransferase inhibitor, hindering the proper activation of any downstream players. The Notch1 protein levels were severely reduced after application of the inhibitor, implying cooperation between Notch1 and the Ras signaling pathway. Following this experiment, Weijzen *et al.* (2002) confirmed that downregulation of Notch1 results in inhibition of Ras activation through treatment with a Notch inhibitor. This discovery is important in developing a treatment for breast cancer because Notch1 levels have been shown to be elevated in breast cancer tissues.

Mittal *et al.* (2009) verified Notch/Ras cooperation through studies done both *in vitro* and *in vivo.* After a sample of human mammary epithelial cells failed to form mammospheres in soft agar, the experiment was repeated in the presence of Ras. Ras cells were infected with Notch1 positive retroviruses, and after a few weeks, clear colonization was observed.

Furthermore, when the Ras/Notch1 combination was inserted *in vivo* into the mammary fat pads of mice, tumors were observed after five weeks. Because tumor formation was observed only when in population with each other and because inhibition of Ras causes Notch downregulation, researchers proposed that the Ras pathway and Notch1 protein are in collaboration redundant.

Notch1 and Jag1 Expression and Poor Prognosis

Much experimental analysis has shown that aberrant activation of the Notch signaling pathway is present in breast cancers. Reedijk *et al.* (2005) tested the prognosis of breast cancers possessing elevated Notch expression. Using widely known clinical software, called Adjuvant!, that holds the nature of patients' previous cancers, scientists were able to correlate Jag1, Notch1, and Notch3 with poor predicted survival. Jag1 had the highest correlation percent, with Notch1 and Notch3 following behind. Based on this evidence, an analysis of tissues expressing these proteins was performed using DNA tissue microarrays and *in situ* hybridization (Reedijk *et al.*, 2005). Interestingly, Jag1 expression alone was found to be a predictor of poor outcome; patients who expressed elevated Jag1 were less likely to survive than those who did not. No significant trends were observed for Notch1 or Notch3 expression alone; however, co-expression of Notch1 and Jag1 were conclusive for poor predicted mortality. Previous experiments have shown a relationship between the two proteins by a co-localization observable through photomicrographs (Reedijk *et al.*, 2005). With the understanding that expression of these proteins is correlated with poor prognosis, urgency is suggested in finding treatment for women with these indications.

Conclusion

Validation of the expression and activation of Notch proteins has brought scientists closer to understanding the role of the Notch signaling pathway in human breast cancer. The deregulation of these proteins is recognized in many breast cancer cell lines, and introducing Notch inhibitors, as well as the re-expression of Numb, has been shown to control this aberrant behavior. Furthermore, scientists have been able to correlate the cooperation of certain Notch proteins with reduced overall survival through experimental evidence as well as previous patient records. Understanding the threat of improperly expressed proteins in

the Notch signaling pathway, how to regulate this expression, and the cell lines at risk for this behavior has opened many doors leading to the treatment of those women affected.

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