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TITLE: Identification of Gene Expression Profiles Associated With Different Types of Breast Adipose and Their Relationship to Tumorigenesis

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14. ABSTRACT Purpose: Having long been thought to function only as an inert energy storage depot, the role of adipose tissue in tumorigenesis has been largely ignored. Improved understanding of the role of adipose in tumorigenesis is crucial given the increasing rates of obesity and the use of autologous fat transfer in breast reconstruction. Scope: Adipose, adjacent to and distant from invasive breast tumors, was laser microdissected from 20 post-menopausal women, and from 20 post-menopausal women with non-malignant breast disease. Gene expression data were generated using U133 2.0 microarrays. Data were analyzed to identify significant patterns of differential expression between adipose classes. Data were validated using qRT-PCR. Major findings: Immune response differs in a gradient fashion between non-malignant, distant and tumor adjacent adipose, with the largest response closest to the tumor. FCGR2A, FOLR2, LGMN, MARCO and NLRP3 were expressed at significantly higher levels and HLA-DQB1 and HLA-DQA1 at significantly lower levels in adipose from invasive breasts compared to non-malignant breasts. MMP9, PLA2G7, RRM2 and SPP1 were expressed at significantly higher levels in adjacent compared to distant adipose. Thus, adipose is not an inert component of the breast microenvironment but plays an active role in tumorigenesis.					
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Table of Contents

	<u>Page</u>
Introduction.....	6
Body.....	7
Key Research Accomplishments.....	9
Reportable Outcomes.....	10
Conclusion.....	11
References.....	13
Appendices.....	14

INTRODUCTION:

Research in the past decade has increased our understanding of how the tumor microenvironment influences tumor development. Breast stroma, which comprises 80% of the normal breast, encompasses fibroblasts, endothelial, smooth muscle, inflammatory and nerve cells, macromolecules of the extracellular matrix (ECM) and adipose¹. Although a number of investigators have characterized molecular changes in the stroma as a whole, or in individual stromal components such as fibroblasts, myoepithelial and endothelial cells², the possible role of adipose, having long been thought to function only as an inert energy storage depot, in tumorigenesis has been largely ignored³. In fact, adipose is an active endocrine organ secreting adipokines, that can directly influence tumor growth⁴, and given that adipose comprises ~50% of the human breast⁵, a complete understanding of how the breast microenvironment contributes to tumorigenesis cannot be achieved without improved understanding of how adipose may contribute to tumor development and progression. In this study, adipose tissue from three different types of breast specimens was characterized using gene expression analysis. By comparing expression of genes from adipose tissue taken from the breasts of postmenopausal women with invasive breast cancer to adipose taken from postmenopausal non-malignant breast specimens, adipose-specific genes involved in tumorigenesis were identified. Similarly, by comparing molecular signatures of adipose located adjacent to invasive breast tumors to those expressed in breast specimens distant to the tumor, genetic pathways actively supporting tumorigenesis were identified. This study serves as the first comprehensive molecular characterization of adipose tissue in malignant and non-malignant breasts.

BODY:*Task 1 Identification and histological evaluation of tissue specimens*

The database was queried to identify all female patients with invasive breast cancer that underwent mastectomy. Patients with BRCA1 or BRCA2 mutations or those with a previous history of cancer were excluded. Patients with multi-focal or multi-centric tumors were also excluded. Given the established protocols to collect research-grade tissue specimens from the four breast quadrants from patients undergoing mastectomy, sufficient distant adipose were available. The rate-limiting factor in collecting these patients was the identification of breast specimens harboring adipose adjacent to the tumor. Moving forward, our recommendation is that when mastectomies are performed, a section of stroma containing adipose as well as a small section of the tumor margin should be collected. In total, 30 patients with both tumor adjacent and distant adipose (Figure 1) were identified. All cases had sufficient areas of adipose to generate usable RNA samples.

Identification of patients with non-neoplastic diagnoses and research-grade specimens containing adipose was more difficult. These patients frequently underwent excisional biopsy, where the smallest surgical areas were removed, and often, the entire specimen was utilized in pathological evaluation. Patients with a history of cancer, BRCA1 or BRCA2 mutations were excluded. Because adipose or fat is not a characteristic listed on the pathology checklist, 73 patients with non-neoplastic diagnoses were identified and requested for use. Of these 73, evaluation of hematoxylin and eosin slides revealed that 27 specimens did not harbor adipose, leaving a total of 46 non-neoplastic tissues for laser microdissection.

Task 2 Gene expression profiling

Sixty stromal specimens from patients with invasive breast cancer (30 adjacent and 30 distant) and 30 non-neoplastic specimens were subjected to laser microdissection to collect pure adipose cell populations. Generally, 2-12 8 μ m sections are sufficient to isolate enough RNA for microarray analysis. With the adipose specimens, however, thicker sections (10 μ m) were cut to keep the adipose sections from falling off the laser capture foil slides. In addition, 10-20 sections were needed to isolate sufficient quantity of RNA. Of the 60 specimens from invasive patients, all generated sufficient quantity and quality RNA for microarray analysis. Of the 46 non-neoplastic specimens, fourteen failed to generate sufficient quantities of RNA. One additional specimen generated sufficient quantity, however, upon evaluation with the 2100 Bioanalyzer, the quality of the specimen was substandard. Thus, RNA of sufficient quantity and quality was isolated from 31 patients with non-neoplastic diagnoses.

RNA from 20 invasive patients (adjacent and distant pairs) and 22 non-neoplastic patients were hybridized to U133A 2.0 arrays. All 42 samples generated gene expression data suitable for data analysis. No modifications to the protocol were needed.

Gene expression data were sent to the collaborating bioinformatician, Ryan van Laar, at ChipDX as .cel files. Affymetrix CEL files were processed using the MAS5.0 algorithm. Individual gene expression values below a normalized value of 10.0 were set to 10.0 and any probe missing from 80% or more of all samples was excluded from further analysis. The normalized profiles were then median-centered across the dataset, to minimize any technical bias present in the dataset. Probe redundancy (i.e. genes represented by >1 probeset) was reduced by selecting the individual probe with the highest mean intensity across all samples. After these steps, there were 9,490 normalized gene expression values per sample. Using a randomized block design, the expression of 9,490 genes was compared between adipose tissue distant to a breast tumor and adipose from individuals without cancer (non-malignant). A p-value threshold of <0.01 was used to identify genes differentially expressed between tissue types (Figure 2). Pathway comparison was used to analyze pre-defined gene sets for differential expression among pre-defined classes. The BioCarta pathway database (<http://www.biocarta.com/genes/index.asp>) was selected and

individual pathways differentially regulated between classes below $P=0.005$ were identified (Tables 1, 2, 3). Permutation analysis was used to determine the significance of the differential expression.

Task 3 qRT-PCR validation of genetic signatures

From the list of differentially expressed genes, twelve were chosen for qRT-PCR validation including NLRP3, LGMN, FCGRA2A, FOLR2, MARCO, HLA-DQB1 and HLA-DQA1, which were differentially expressed between invasive distant adipose and non-neoplastic adipose, and RRM2, PLA2G7, ADAMDEC1, SPP1 and MMP9, which were differentially expressed between adjacent and distant adipose from patients with invasive breast cancer. Validation was performed in the original specimens used to generate microarray data (adjacent $n=20$, distant $n=20$, non-neoplastic $n=22$) as well as adipose samples from an additional 10 women with invasive disease and 9 women with non-neoplastic disease. Given the small starting quantities of RNA, sufficient non-amplified RNA was not available from the original cases, thus RNA from the additional out-of-sample adipose specimens was subjected to two-rounds of amplification and labeled, so that all RNA specimens utilized in the validation studies were in equivalent states.

Relative quantification of gene expression levels was determined using the Comparative C_t method and the medians of the Comparative C_t values for each type of adipose were compared using a Mann-Whitney U test (<http://elegans.swmed.edu/~leon/stats/utest.html>) to determine if the relative fold change was significantly different between the two groups.

Preliminary results from this project were submitted to the AACR annual meeting⁶ and will be presented as a poster (Appendix 1). Final results will be submitted to the SABCS meeting⁷ and a manuscript describing the results is currently *in preparation*⁸, to be submitted to *Cancer Research*.

KEY RESEARCH ACCOMPLISHMENTS: Bulleted list of key research accomplishments emanating from this research.

- Development of protocols to effectively generate microarray-based gene expression data from breast adipose. Although adipose is a major component of the breast, the large size of fat cells renders regions of adipose tissue less cellular than tumor areas. In addition, the high lipid content of adipose can make it difficult to work with from frozen sections. We successfully demonstrated that sufficient RNA can be isolated from breast adipose to generate microarray data.
- Identification of gene expression differences in tumor-adjacent adipose compared to distant or non-malignant adipose. B- and T-cell immunity pathways are the most different between tumor-adjacent and distant or non-malignant adipose, with the highest response in adjacent adipose, followed by distant adipose and the lowest response was seen in adipose from non-malignant breast specimens. A number of genes differentially expressed between tumor adjacent and distant and non-malignant adipose including RRM2, PLA2G7, ADAMDEC1, SPP1 and MMP9 in adjacent compared to distant and EGFL6, ITGB2 and PIP, in adjacent compared to non-malignant adipose. These genes which may contribute to tumorigenesis by stimulating cellular proliferation, degrading the extracellular matrix, altering cellular adhesion, increasing migration and invasion and enhancing inflammation.
- Identification of gene expression differences in distant adipose from breasts with invasive tumors compared to adipose from non-malignant breasts. Genes such as NLRP3, LGMN, FCGR2A, and MARCO, which are involved in processes such as inflammation and immune response, are expressed at significantly higher levels in adipose from breasts with invasive disease, while HLA-DQA1 and HLA-DQB1, involved in immune response, are expressed at significantly higher levels in non-neoplastic breast adipose. These data suggest that the predominant difference between these two types of non-tumor adjacent adipose is the heightened immunological response in the invasive breast.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

1. An abstract reporting the preliminary results (Appendix 2) was accepted to the AACR annual meeting (see Appendix 1 for poster).
2. An abstract reporting the final results (Appendix 3) was submitted to the SABCS annual meeting (to be held December 2012).
3. A manuscript describing the results is currently *in preparation* with the hopes of acceptance to *Cancer Research*.

CONCLUSION: Summarize the results to include the importance and/or implications of the completed research and when necessary, recommend changes on future work to better address the problem. A "so what section" which evaluates the knowledge as a scientific or medical product shall also be included in the conclusion of the report.

In conclusion, we have demonstrated that it is possible to generate microarray-based gene expression from breast adipose. This is important as breast adipose, which is a major component of the breast microenvironment, has not been well-studied for its possible role in tumor development and progression.

Three types of gene expression analyses were performed: tumor-adjacent to matched distant adipose, tumor-adjacent to non-malignant and distant to non-malignant. The first finding is that there is a gradient immune response across the three types of adipose, with the strongest response in the tumor-adjacent adipose, with a weaker response in the distant adipose and the weakest response in adipose from non-malignant breasts. This response may be a biological response of the adipose to the tumor; however, it is also possible that this heightened immune response is the result of the surgical trauma from the original incisional biopsy. All of the specimens collected from patients with invasive breast cancer had undergone a diagnostic biopsy followed by a mastectomy. In contrast, non-malignant specimens were collected by excisional biopsy; a second surgery was not performed, thus adipose from non-malignant breasts may not demonstrate a surgery-induced immune response. Methods to overcome these differences are not practical within the Clinical Breast Care Project (CBCP): women suspected of having non-malignant disease could be asked to have an incisional biopsy done before the excisional biopsy, however, CBCP protocols mandate that excess tissues beyond what is clinically warranted may be collected only for research purposes. Alternatively, patients with invasive breast cancer who have undergone mastectomy without incisional biopsy, such as those undergoing prophylactic mastectomy with an incidental finding of breast cancer, could be evaluated. These patients are extremely rare with <5 instances over the 10 years of the program.

Although a gradient immune response was the predominant pathway difference between types of fat, gene expression differences between tumor-adjacent and both distant and non-malignant adipose suggest that tumor-adjacent adipose is a more active adipose, expressing a number of genes that are involved in tumor-related processes such as growth, proliferation, degradation of the extracellular matrix, and angiogenesis. Because these genetic changes are detected not only between adipose specimens from different individuals (tumor-adjacent and non-malignant) but between adipose specimens from the same breast (tumor-adjacent and matched distant), these molecular differences are likely reflective of differences in function of adipose adjacent to the tumor, rather than inter-individual differences or the effect of previous surgical injury.

What do these results mean? Numerous studies have investigated molecular changes in the stroma as a whole, as well as in individual components of the stroma, however, to our knowledge this is the first evaluation of gene expression changes of breast-related adipose. Because adipose can account for ~50% of the human breast, biological understanding of the role of stroma in tumorigenesis is not complete without a better understanding of the function of adipose. Thus, with the identification of differentially expressed genes such as RRM2 and MMP9 in tumor-adjacent adipose, we have demonstrated that adipose differs based on proximity to the tumor and identified genes that may be important in tumorigenesis. Additionally, these results provide the first molecular targets for the development of new, stromal-based molecular therapeutics for the treatment of breast cancer.

Future directions: These data demonstrate for the first time that gene expression levels differ in breast adipose, depending on presence of and proximity to tumor cells. Obesity is a risk factor in post-menopausal, but not pre-menopausal, women. All adipose samples in this study were procured from post-menopausal women, thus, it is important to evaluate similar types of adipose in pre-menopausal women to determine whether tumor-adjacent adipose in younger women also demonstrates similar gene expression changes suggesting a role in tumorigenesis and whether there are differences in gene expression in adipose between pre- and post-menopausal women which may be affected by changes in the hormonal milieu. A second critical study would be to evaluate gene expression differences in adipose between patients who are healthy weight, overweight or obese. Evaluation of adipose between women of healthy weight and obese women would provide data as to whether excess adiposity alters the molecular biology of breast adipose, creating a more hospitable environment for tumorigenesis. Given the increasing obesity epidemic worldwide, determination of whether breast adipose from obese women contributes to tumor etiology will be an important contribution to the development of public health policies aimed at achieving or maintaining a healthy weight.

REFERENCES: List all references pertinent to the report using a standard journal format (i.e. format used in *Science*, *Military Medicine*, etc.).

- (1) Shekhar MP, Pauley R, Heppner G. Host microenvironment in breast cancer development: extracellular matrix-stromal cell contribution to neoplastic phenotype of epithelial cells in the breast. *Breast Cancer Res* 2003;5(3):130-135.
- (2) Allinen M, Beroukhi R, Cai L et al. Molecular characterization of the tumor microenvironment in breast cancer. *Cancer Cell* 2004;6(1):17-32.
- (3) Iyengar P, Espina V, Williams TW et al. Adipocyte-derived collagen VI affects early mammary tumor progression in vivo, demonstrating a critical interaction in the tumor/stroma microenvironment. *J Clin Invest* 2005;115(5):1163-1176.
- (4) Maccio A, Madeddu C, Mantovani G. Adipose tissue as target organ in the treatment of hormone-dependent breast cancer: new therapeutic perspectives. *Obes Rev* 2009;10(6):660-670.
- (5) Lejour M. Evaluation of fat in breast tissue removed by vertical mammoplasty. *Plast Reconstr Surg* 1997;99(2):386-393.
- (6) Field LA, Deyarmin B, van Laar R, Shriver CD, Ellsworth RE. Identification of gene expression profiles associated with different types of breast adipose and their relationship to tumorigenesis. *Proceed.AACR* . 2012.
- (7) Field LA, Deyarmin B, van Laar R, Hooke JA, Shriver CD, Ellsworth RE. Molecular drivers of adipogenotoxicosis in breast tumor-associated adipose. *SABCS* submitted. 2012.
- (8) Field LA, Deyarmin B, van Laar R, Hooke JA, Shriver CD, Ellsworth RE. Identification of gene expression profiles associated with different types of breast adipose and their relationship to tumorigenesis. *in preparation* . 2012.

APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, study questionnaires, and surveys, etc.

SUPPORTING DATA: All figures and/or tables shall include legends and be clearly marked with figure/table numbers.

Table 1: BioCarta pathways with significant differential expression between adjacent and non-malignant adipose.

Biocarta Pathway	Pathway description	Number of genes
h_blymphocytePathway	B Lymphocyte Cell Surface Molecules	15
h_ctla4Pathway	The Co-Stimulatory Signal During T-cell Activation	17
h_eosinophilsPathway	The Role of Eosinophils in the Chemokine Network of Allergy	7
h_tcraPathway	Lck and Fyn tyrosine kinases in initiation of TCR Activation	10
h_tcytotoxicPathway	T Cytotoxic Cell Surface Molecules	12
h_th1th2Pathway	Th1/Th2 Differentiation	18
h_thelperPathway	T Helper Cell Surface Molecules	11
h_classicPathway	Classical Complement Pathway	9
h_pepiPathway	Proepithelin Conversion to Epithelin and Wound Repair Control	5
h_il10Pathway	IL-10 Anti-inflammatory Signaling Pathway	15
h_mhcPathway	Antigen Processing and Presentation	16
h_compPathway	Complement Pathway	11
h_CSKPathway	Activation of Csk by cAMP-dependent Protein Kinase Inhibits Signaling through the T Cell Receptor	19
h_bbcellPathway	Bystander B Cell Activation	12
h_asbcellPathway	Antigen Dependent B Cell Activation	13
h_il5Pathway	IL 5 Signaling Pathway	8
h_plateletAppPathway	Platelet Amyloid Precursor Protein Pathway	12
h_plcPathway	Phospholipase C Signaling Pathway	13
h_sppaPathway	Aspirin Blocks Signaling Pathway Involved in Platelet Activation	17
h_inflamPathway	Cytokines and Inflammatory Response	14
h_myosinPathway	PKC-catalyzed phosphorylation of inhibitory phosphoprotein of myosin phosphatase	11
h_npp1Pathway	Regulators of Bone Mineralization	9
h_vitCBPathway	Vitamin C in the Brain	14
h_nkcellsPathway	Ras-Independent pathway in NK cell-mediated cytotoxicity	19
h_eradPathway	ERα associated degradation (ERAD) Pathway	29
h_ace2Pathway	Angiotensin-converting enzyme 2 regulates heart function	11
h_cblPathway	CBL mediated ligand-induced downregulation of EGF receptors	15
h_glycolysisPathway	Glycolysis Pathway	8
h_ctbp1Pathway	SUMOylation as a mechanism to modulate CtBP-dependent gene responses	13

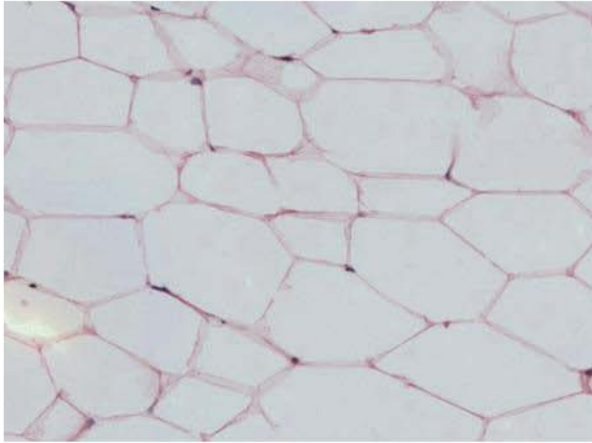
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h_mhcPathway	Antigen Processing and Presentation	16
h_compPathway	Complement Pathway	11
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h_bbcellPathway	Bystander B Cell Activation	12
h_asbcellPathway	Antigen Dependent B Cell Activation	13
h_il5Pathway	IL 5 Signaling Pathway	8
h_plateletAppPathway	Platelet Amyloid Precursor Protein Pathway	12
h_plcPathway	Phospholipase C Signaling Pathway	13
h_sppaPathway	Aspirin Blocks Signaling Pathway Involved in Platelet Activation	17
h_inflamPathway	Cytokines and Inflammatory Response	14
h_myosinPathway	PKC-catalyzed phosphorylation of inhibitory phosphoprotein of myosin phosphatase	11
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h_cblPathway	CBL mediated ligand-induced downregulation of EGF receptors	15
h_glycolysisPathway	Glycolysis Pathway	8
h_ctbp1Pathway	SUMOylation as a mechanism to modulate CtBP-dependent gene responses	13

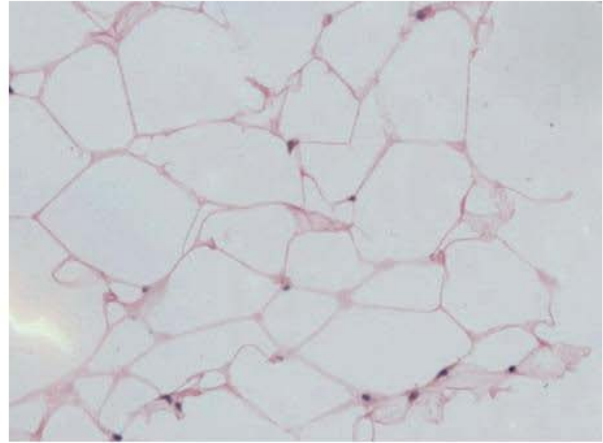
Table 3: Differentially regulated BioCarta pathways between paired specimens of adjacent and distant adipose tissue

Biocarta Pathway	Pathway description	Number of genes
h_tcytotoxicPathway	T Cytotoxic Cell Surface Molecules	12
h_thelperPathway	T Helper Cell Surface Molecules	11
h_blymphocytePathway	B Lymphocyte Cell Surface Molecules	15
h_eradPathway	ER-associated degradation (ERAD) Pathway	29
h_ctla4Pathway	The Co-Stimulatory Signal During T-cell Activation	17
h_CSKPathway	Activation of Csk by cAMP-dependent Protein Kinase Inhibits Signaling through the T Cell Receptor	19
h_compPathway	Complement Pathway	11
h_tcraPathway	Lck and Fyn tyrosine kinases in initiation of TCR Activation	10
h_d4gdiPathway	D4-GDI Signaling Pathway	18
h_eosinophilsPathway	The Role of Eosinophils in the Chemokine Network of Allergy	7
h_mhcPathway	Antigen Processing and Presentation	16
h_caspasePathway	Caspase Cascade in Apoptosis	31
h_actinYPathway	Y branching of actin filaments	13
h_fbw7Pathway	Cyclin E Destruction Pathway	11
h_il18Pathway	IL 18 Signaling Pathway	6
h_At1rPathway	Angiotensin II mediated activation of JNK Pathway via Pyk2 dependent signaling	39
h_skp2e2fPathway	E2F1 Destruction Pathway	12
h_Ccr5Pathway	Pertussis toxin-insensitive CCR5 Signaling in Macrophage	28

Figure 1. Tumor-adjacent and distant adipose from a patient with invasive breast cancer. The image on the left is adipose adjacent to an ER+/PR-/HER2-, moderately-differentiated IDCA. The image on the right is distant adipose located 4-cm from the tumor.



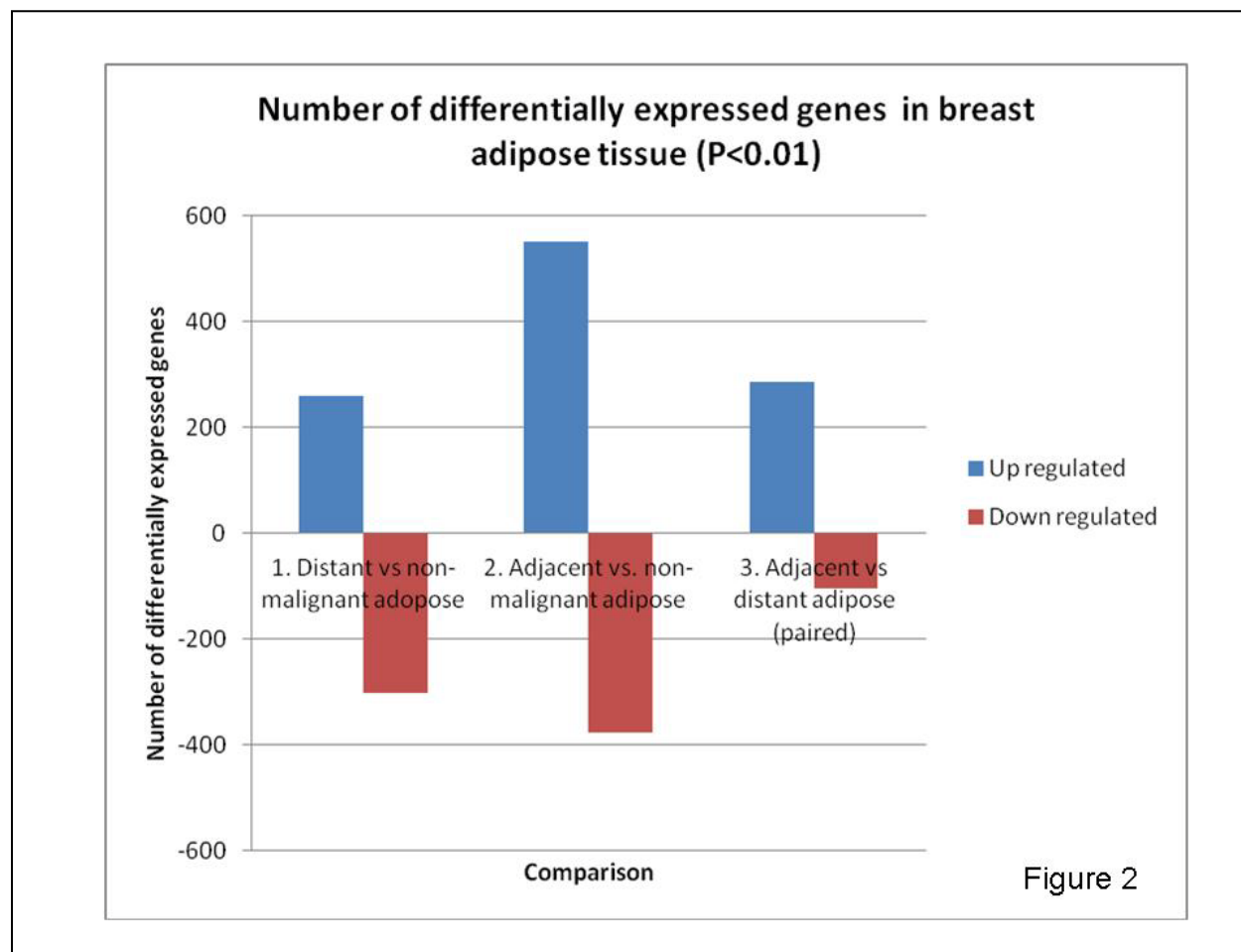
Adjacent adipose



Distant adipose

Figure 1

Figure 2. Chart of number of genes differentially expressed between tumor-adjacent, distant and non-malignant adipose. From the 9,490 normalized, non-redundant genes, a randomized block design was used to identify differentially expressed genes using a p-value threshold of <0.01 . In the comparison between tumor-adjacent and distant adipose, 391 differentially expressed genes were identified, between tumor-adjacent and non-malignant 1,092 genes were identified and between distant and non-malignant, 562 differentially expressed genes were identified.



Identification of gene expression profiles associated with different types of breast adipose and their relationship to tumorigenesis

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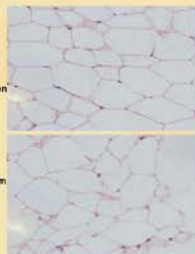
LA Field¹, R van Laar², B Deyarmin³, CD Shriver³, RE Ellsworth⁴

¹Windber Research Institute, Windber, PA; ²ChpDx, New York, NY; ³Walter Reed National Military Medical Center, Bethesda, MD; ⁴Henry M Jackson Foundation, Windber, PA

Background

Research over the past decade has shown the importance of the stroma in tumorigenesis; however, having long been thought to function only as an inert energy storage depot, the role of adipose tissue in tumorigenesis has been largely ignored. Improved understanding of the role of adipose in tumorigenesis is crucial given the increasing rates of obesity and the use of autologous fat transfer in breast reconstruction.

Figure 1: Adipose from a patient with invasive breast cancer. The image on the top is adipose adjacent to a ER+/PR-/HER2-, moderately-differentiated IDC. The image on the bottom is distant adipose located 4-cm from the tumor.



Methods

• Adipose, adjacent to and distant from invasive breast tumors (Figure 1), was laser microdissected from 20 post-menopausal women, and from 22 post-menopausal women with non-malignant breast disease.

• Gene expression data were generated using U133A 2.0 microarrays.

• After quality control and visualization steps, the data were analyzed to identify significant patterns of differential expression between adipose classes, at the individual gene and molecular pathway level.

BioCarta Pathway	Pathway description	Number of genes
1. p53Pathway	The p53 pathway is a key pathway in the regulation of cell growth and death.	28
2. WntPathway	The Wnt pathway is a key pathway in the regulation of cell growth and differentiation.	28
3. NotchPathway	The Notch pathway is a key pathway in the regulation of cell growth and differentiation.	28
4. HedgehogPathway	The Hedgehog pathway is a key pathway in the regulation of cell growth and differentiation.	28
5. TGF-betaPathway	The TGF-beta pathway is a key pathway in the regulation of cell growth and differentiation.	28
6. JAK-STATPathway	The JAK-STAT pathway is a key pathway in the regulation of cell growth and differentiation.	28
7. NF-kappaBPathway	The NF-kappaB pathway is a key pathway in the regulation of cell growth and differentiation.	28
8. MAPKPathway	The MAPK pathway is a key pathway in the regulation of cell growth and differentiation.	28
9. PI3KPathway	The PI3K pathway is a key pathway in the regulation of cell growth and differentiation.	28
10. mTORPathway	The mTOR pathway is a key pathway in the regulation of cell growth and differentiation.	28

Table 1: Differentially regulated BioCarta pathways between paired specimens of adjacent and distant adipose tissue

Results

• Pathway analysis revealed that immune response differs between non-malignant, distant and tumor adjacent adipose (Tables 1 and 2); this response is seen as a gradient with the largest response closest to the tumor.

• Gene expression differed significantly in adipose from invasive compared to non-malignant breasts with FCGR2A, FOLR2, LGMN, MARCO and NLRP3 (Figure 2) expressed at significantly higher levels and HLA-DQB1 and HLA-DQA1 at significantly lower levels in adipose from invasive breasts (Figure 2).

• Within the invasive breasts, MMP9, PLA2G7, RRM2 and SPP1 were expressed at >3-fold higher levels in adjacent compared to distant adipose.

BioCarta Pathway	Pathway description	Number of genes
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7. NF-kappaBPathway	The NF-kappaB pathway is a key pathway in the regulation of cell growth and differentiation.	28
8. MAPKPathway	The MAPK pathway is a key pathway in the regulation of cell growth and differentiation.	28
9. PI3KPathway	The PI3K pathway is a key pathway in the regulation of cell growth and differentiation.	28
10. mTORPathway	The mTOR pathway is a key pathway in the regulation of cell growth and differentiation.	28

Table 2: Results from BioCarta pathway comparison between distant and non-malignant breast adipose tissue.

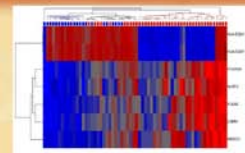


Figure 2: Heat map of 7 genes differentially expressed between adipose from invasive breasts (red squares) and non-malignant breasts (blue squares).

Conclusion

Gene expression levels differ in breast adipose, depending on presence of or proximity to tumor cells. Adipose adjacent to the tumor demonstrated the largest immune response; this response may reflect a reaction to surgical insult from the original biopsy, however, response to surgical injury has been associated with increased ability to metastasize. In addition, within breasts with invasive breast cancer, genes involved in cellular proliferation, degradation of the extracellular matrix and angiogenesis were expressed at higher levels in adjacent compared to distant adipose. Together, these data suggest that adipose is not an inert component of the breast microenvironment but plays an active role in tumorigenesis.

Appendix 1. Poster from the AACR annual meeting (March 31-April 5, 2012, Chicago)

Appendix 2. Abstract submitted to and presented at AACR annual meeting

Identification of Gene Expression Profiles Associated with Different Types of Breast Adipose and Their Relationship to Tumorigenesis

Lori Field, Brenda Deyarmin, Ryan van Laar, Craig Shriver, Rachel Ellsworth

Background: Research over the past decade has shown the importance of the stroma in tumorigenesis, however, having long been thought to function only as an inert energy storage depot, the role of adipose tissue in tumorigenesis has been largely ignored. Improved understanding of the role of adipose in tumorigenesis is crucial given the increasing rates of obesity and the use of autologous fat transfer in breast reconstruction.

Methods: Adipose, adjacent to and distant from invasive breast tumors, was laser microdissected from 20 post-menopausal women, and from 20 post-menopausal women with non-malignant breast disease. Gene expression data were generated using U133 2.0 microarrays. After quality control and visualization steps, the data were analyzed to identify significant patterns of differential expression between adipose classes, at the individual gene and molecular pathway level.

Results: Pathway analysis revealed that immune response differs between non-malignant, distant and tumor adjacent adipose; this response is seen as a gradient with the largest response closest to the tumor. Gene expression differed significantly in adipose from invasive compared to non-malignant breasts with FCGR2A, FOLR2, LGMN, MARCO and NLRP3 expressed at significantly higher levels and HLA-DQB1 and HLA-DQA1 at significantly lower levels in adipose from invasive breasts. Within the invasive breasts, MMP9, PLA2G7, RRM2 and SPP1 were expressed at >3-fold higher levels in adjacent compared to distant adipose.

Conclusions: Gene expression levels differ in breast adipose, depending on presence of or proximity to tumor cells. Adipose adjacent to the tumor demonstrated the largest immune response; this response may reflect a reaction to surgical insult from the original biopsy; however, response to surgical injury has been associated with increased ability to metastasize. In addition, within breasts with invasive breast cancer, genes involved in cellular proliferation, degradation of the extracellular matrix and angiogenesis were expressed at higher levels in adjacent compared to distant adipose. Together, these data suggest that adipose is not an inert component of the breast microenvironment but plays an active role in tumorigenesis.

Appendix 3. Abstract submitted to SABCS (to be held December 2012).

Molecular drivers of adipogenotoxicosis in breast tumor-associated adipose

Lori Field, Brenda Deyarmin, Ryan van Laar, Jeff Hooke, Craig Shriver, Rachel Ellsworth

Background: Having long been thought to function only as an inert energy storage depot, the role of adipose tissue in tumorigenesis has been largely ignored; however, adipose is an active endocrine organ that can directly influence tumor growth. Improved understanding of the role of adipose in tumorigenesis is crucial given the association between obesity and breast cancer risk in post-menopausal women, increasing rates of obesity and use of autologous fat transfer in breast reconstruction.

Methods: Adipose, adjacent to and distant from (>3 cm from the closest tumor margin) invasive breast tumors, was laser microdissected from 20 post-menopausal women, and from 22 post-menopausal women with non-malignant breast disease. Gene expression data were generated using U133A 2.0 microarrays. Data were analyzed to identify significant patterns of differential expression between adipose classes at the individual gene and molecular pathway level. Gene expression differences were validated using qRT-PCR in an additional set of 29 specimens.

Results: SPP1, RRM2, MMP9 and PLA2G7 were expressed at >3-fold ($P < 0.01$) higher levels in adjacent adipose compared to distant adipose from the same breast. A number of immune response genes including MARCO, FABP7, ELF5, MYBPC1, MMP7, CLDN8, HLA-DQB1 and HLA-DQA1 were differentially expressed in distant adipose compared to adipose from non-malignant breasts. The most significant gene expression differences were detected between tumor-adjacent and non-malignant adipose with >3-fold higher expression of EGFL6 and ITGB2 and >3-fold lower levels of PIP, which are involved in growth, proliferation, and cellular adhesion in adjacent compared to non-malignant adipose. Pathway analysis revealed that immune response differs between non-malignant, distant and tumor-adjacent adipose with an enhanced B- and T-cell response detected in adjacent compared to distant or non-malignant adipose. Inflammatory response as well as DNA transcription and replication pathways were differentially expressed in distant compared to non-malignant adipose.

Conclusions: Gene expression levels differ in breast adipose depending on presence of and proximity to tumor cells. Adipose adjacent to the tumor demonstrated the largest immune response, supporting the idea of adipogenotoxicosis, which through pro-inflammatory and genotoxic responses, promotes tumor development. In addition, genes involved in cellular proliferation, degradation of the extracellular matrix and angiogenesis are differentially expressed in adjacent compared to distant or non-malignant adipose, thus tumor-adjacent adipose may be contributing to the growth and invasion of the primary tumor. These data thus suggest that adipose is not an inert component of the breast microenvironment but plays an active role in tumorigenesis.