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Sections from 90 different ductal breast carcinomas were stained with monoclonal antibodies specific for different oligosaccharide sequences that are expressed unusually by cancer cells. Both immunofluorescence techniques and immuno streptavadin-biotin techniques were used to evaluate the fraction of tumor cells that are positive and the relative amount of reaction product as measured using an image processing system. Continuing studies of additional tumors are in progress, however preliminary analysis of the available data has shown that the extended Le^a-Le^x oligosaccharide is a statistically significant indicator of poor prognosis following surgical removal of small, node-negative ductal breast carcinomas. Additional studies have suggested that the variable expression of this marker oligosaccharide in different cells of the same tumor is attributable at least in part to differing cell-cell interactions in the tumor.

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David E Bellgard 9-12-95

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INTRODUCTION

Previous studies from several laboratories have shown that the prognosis is generally favorable for breast cancer patients who have a small < 5 cm node-negative tumor (see for review ref. 1). After surgery, relapse during a 5-10 year period occurs in less than 20% of these so called low-risk patients. Yet oncologists are faced with a difficult decision in managing these patients, since they have no clear way of identifying the 20% who will relapse. Thus the patients who could benefit most from adjuvant chemotherapy cannot be identified and equally important, the patients who don't require post-surgical adjuvant therapy cannot be unambiguously identified. The purpose of the present research is to devise an approach for identifying the low-risk patients who are at risk.

The general goal of these studies is to determine if there are specific combinations of oligosaccharide markers on breast cancer cells that are useful in predicting the post surgical prognosis of low-risk node-negative breast cancer patients. Useful prognostic markers identified from these studies would then be combined with other known prognostic markers in an attempt to assemble a set of markers which could indicate with highest specificity and sensitivity the patients who are at greatest risk for relapse.

We are studying a large group of tumor breast tumor specimen obtained from a collection of the Danish Breast Cancer Cooperative Group which is a nationwide surveillance and research program (2). All specimen are from women who had low-risk node negative ductal breast carcinomas and who had surgery 5-15 years previously and who have been closely followed since surgery. None of the women had chemotherapy, so that the prognosis is unaffected by other post-surgical interventions. A panel of well characterized monoclonal antibodies with known specificity for specific oligosaccharides is employed to define the cell surface oligosaccharides, proteolytic activities (such as Cathepsins) and protease inhibitors associated with the tumor cells. After completing the analysis, the relapse history of the patients will be compared with the different molecular markers using Cox's proportional hazards model to identify statistically significant independent markers of prognosis. It will then be possible to select different combinations of markers to attempt to improve specificity and sensitivity by using a panel of prognostic markers.

Additional related research is seeking to identify the glycosyltransferase activities that are abnormally expressed in breast cancer cells that lead to aberrant expression of specific marker oligosaccharides. Here we are attempting to clone cDNAs recognizing genes that are overexpressed in cells overexpressing the Le^a-Le^x oligosaccharide, which is the best prognostic indicator which we have identified.

This research is still in progress and was planned to be in progress at this stage. Therefore conclusions and detailed summaries of the data to date are premature. However the preliminary review of the data provided below indicates that there is a statistically significant association of the Le^a-Le^x oligosaccharide and poor prognosis of low-risk ductal breast carcinomas.

BODY

The following monoclonal antibodies (Mabs) specific for the designated oligosaccharides were applied to multiple paraffin sections of the first 84 tumor specimen to be examined (see the following page). We have used double-label immunofluorescence microscopy techniques that apply fluorescene and rhodamine conjugated antibodies simultaneously so that the distribution of two different oligosaccharides can be simultaneously determined in the same tumor section (3,4). Fluorescence images are analyzed using the Quantimet 500+ Image Processing System to define both the fraction of tumor cells that are positive, (above a defined baseline), and the intensity of the reaction relative to positive and negative control cells that are processed at the same time. Thus it becomes possible to estimate the relative amounts of specific oligosaccharides expressed primarily as cell surface components on the tumor cells and also to quantitate the fraction of the total tumor cells expressing the oligosaccharide. Remarkable heterogeneity has been noted among different tumors for these oligosaccharides. For example Mab 43-9F recognizing the extended Le^a-Le^x oligosaccharide reacts with nearly 100% of the cells of a few tumors and about 30% of

Table 1. Oligosaccharides Studied in This Investigation

Oligosaccharide Abreviation	Structure	Monoclonal Antibody
Le^a	Galβ1→3GlcNAcβ1→3Galβ1→4Glcβ1--- 4 ↓ Fucα1	CO-514
Sialyl- Le^a	NeuAcα2→3Galβ1→3GlcNAcβ1→3Galβ1→4Glcβ1--- 4 ↓ Fucα1	NS19-9
Le^x	Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1--- 3 ↓ Fucα1	P12
Le^y	Fucα1→2Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1--- 3 ↓ Fucα1	F-3
Le^a -Le^x	Galβ1→3GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1--- 4 3 ↓ ↓ Fucα1 Fucα1	43-9F
Tn	Galβ1→3GalNAcα1→O--Ser/Thr	IE3
Sialyl-Tn	NeuAcα2→Galβ1→3GalNAcα1→O--Ser/Thr	TKH2
Tx	Galβ1→4GalNAcα1→O--Ser/Thr	3C9

other tumors are completely negative, but the majority have a fraction of cells that are positive, ranging from 1 to 100 % of the tumor cells in a section. Sections from the same set of tumors as well as 36 other tumors have also been studied by our collaborator Dr. Johan Andersen in Denmark using similar Mabs and immunoperoxidase in streptavidin-biotin staining methods to estimate the fraction of positive tumor cells and the intensity of reaction product.

As was planned in the original grant application, this research in progress, and we still have about 150 tumors to examine. It was decided that the analysis would be more efficient if we combined the work in goals 1 and 2 so that we could simultaneously determine the presence of Le^a-Le^x oligosaccharide and all the other oligosaccharides in specimens from the same tumors. This avoids the necessity of going back and reexamining the same specimen. Thus the work of goal 1 anticipated to be completed during year 1 will not be competed until that of goal 2 is also done. We have not yet statistically analyzed all of the data that have been collected, but statistical analysis using the proportional hazards model (5) applied to part of the data has already indicated that there is prognostic significance of the extended Le^a-Le^x. Patients with tumors that are negative for this oligosaccharide have less than half the chance of relapsing during the 5-12 year post-surgical period than patients who have positive tumor cells ($P > 0.005$). Also among the patients who relapse there is a correlation between the fraction of tumor cells that are positive for extended Le^a-Le^x and the time after surgery before relapse occurs. Tumors with the highest fraction of positive cells are more likely to experience earlier relapse (Fig. 1). More rigorous statistical analyses and combined analysis of different prognostic indicators will be available when more tumors have been analyzed.

As already discussed, we have been impressed by the heterogeneity of expression of extended Le^a-Le^x and other oligosaccharides by cells in the same tumor. This sort of heterogeneity for cell surface oligosaccharides, as well as other molecular markers has been previously reported, but the reason for the differing expression is not clear. An understanding of this variability might contribute to the goals of the present project, because the preliminary results just reviewed are indicating that there may be prognostic significance to the fraction of tumor cells that are positive for specific oligosaccharides. In this regard we have observed that tumor cells, grown in tissue culture, often display the same kind of heterogeneity, even when the cultured cells are derived from a single clone. During the past year we began a systematic study of this variable expression, which was triggered by the chance observation that the heterogeneity of expression seemed to depend on the density at which tumor cells grow in culture. In detailed studies this observation has now confirmed. For example certain tumor cells do not express when they grow as separated cells at low densities, but begin to express when small colonies of about 25 cells are formed, then when cells become confluent more uniform expression occurs. Detailed studies have confirmed the hypothesis that the expression of certain oligosaccharides associated with mucins and other glycoproteins are influenced by cell-cell interactions among tumor cells. These findings have now been submitted for publication. To our knowledge these finding are the first reported indication of protein structure via glycosylation being controlled by cell-cell contacts.

The project designed to clone cDNAs specifying the glycosyltransferase required to extend Le^a into Le^a-Le^x oligosaccharides (objective #5) is progressing. We constructed a p-bluescript cDNA library made from total mRNA of the human lung cancer cell line NU6-1. The NU6-1 line was previously characterized in this laboratory and is known to overexpress the Le^a-Le^x oligosaccharide (3). A similar library was also made from cDNA homologous to mRNA of human lung cancer cell line NE-18. NE-18 is a variant clone selected from NU6-1 that makes no detectable Le^a-Le^x. These p-bluescript libraries have large cDNA inserts (weight average 3Kb). Subtractive hybridization procedures were employed using the cloned cDNAs from the NE-18 library as driver against cDNAs cloned from NU6-1 to select p-bluescript clones containing cDNA rich in NU6-1 but deficient in NE-18. cDNAs selected by this process were amplified, cut out, end-labeled, and used as probe of p-bluescript plaques to verify that they represent abundant sequences in NU6-1 that are very rare in NE-18.

Candidate cDNAs obtained from the above selection have been partially sequenced (500 bp sequences) to identify those from unknown genes. One common clone was that coding for the previously identified

decay accelerating factor (sequence accession No, gb M31516) (see ref. 6). Several other sequences were also identified that are not found in the gene bank. These are candidates to code for the critical glycosyltransferase.

The candidate cDNA inserts were subcloned into the mammalian expression vector pcDNA3 (also carrying the neomycin resistance gene). These plasmids were transfected into NE-18 cells (that express no detectable extended Le^a-Le^x), transformants selected in the presence of neomycin and screened using the 43-9F monoclonal antibody that recognizes extended Le^a-Le^x. Preliminary findings indicated that many transformants are positive for extended Le^a-Le^x as expected if the cDNA codes for the critical glycosyltransferase or some other factor controlling the synthesis of this oligosaccharide marker. Continuing studies will seek to verify these findings and also to exploit the panning method (8) for selection of transformants carrying cloned copies of the critical glycosyltransferases.

CONCLUSIONS

Results to date have indicated that the prognosis is poorer when low-risk small ductal breast carcinomas are positive for extended Le^a-Le^x oligosaccharide. Moreover the period of time between surgery and relapse seems to be reduced when tumors have a higher fraction of cells expressing the extended Le^a-Le^x oligosaccharide We also showed that the expression of the extended Le^a-Le^x oligosaccharide is strongly influenced by cell-cell interactions among tumor cells and that the oligosaccharide modifications on tumor associated mucins are influenced by the cell-cell interactions. Candidate cDNA sequences have also been cloned that may code for factors essential for the synthesis of extended Le^a-Le^x in cancer cells

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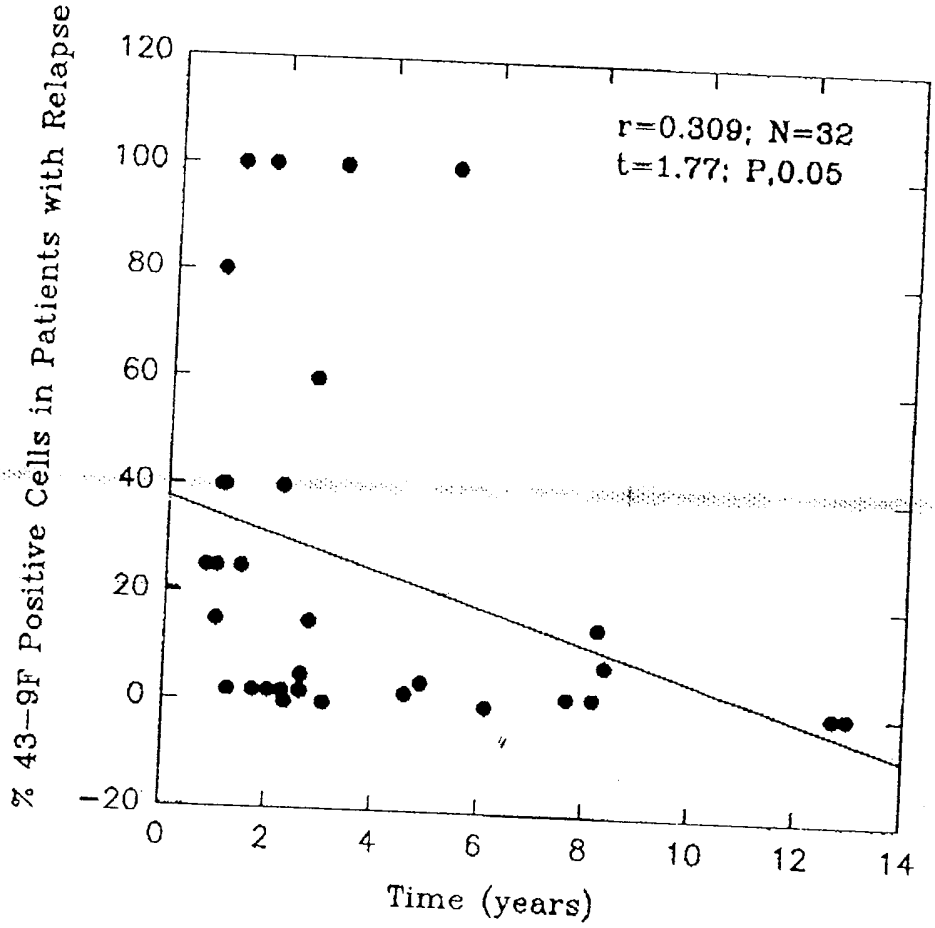

Figure 1. Correlation Between Time of Relapse after Surgery and Fraction of Primary Tumor Cells that are Positive for the Extended Le^a -Le^x Oligosaccharide Recognized by Mab 43-9F. The figure shows an analysis of 31 patients who relapsed during the period 0.5 to 14 years following surgery.