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14. ABSTRACT Seladin-1 maps to the human chromosome region 1p31-1p32 that shows frequent loss of heterozygosity (LOH) in human breast tumors. Publicly available data (Entrez, GEO) also show a significant variability between levels of Seladin-1 expression in breast cancer cell lines and normal breast epithelium. Is Seladin-1 a tumor suppressor on 1p31-1p32 that is involved in breast cancer? Our concept is that it is. In order to determine if Seladin-1 is a TSG involved in breast cancer we propose to: 1) Identify possible Seladin-1 mutations in primary breast tumors. 2) Investigate the alterations of Seladin-1 expression in breast cancer cells. 3) Perform functional assays on tumor-specific Seladin-1 mutants. Accordingly, we amplified Seladin-1 exons from from 40-60 breast tumor genomic DNA samples (obtained from tissue banks or commercial sources), followed by the sequence analysis of the open reading frames. As a result, no missense or nonsense mutations were detected. We found that expression of Seladin-1 significantly varies between different breast cancer cell lines, and in one such line, MDAMB- 231, is significantly below expression in normal breast epithelium.					
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INTRODUCTION:

Seladin-1 has been described recently as a novel regulator of p53, a major human tumor suppressor gene (TSG; [1]). Seladin-1 acts to counteract Mdm2 and promote accumulation of p53 in response to oncogenes and oxidative stress [1]. P53 is implicated in breast cancer progression, but it is mutated only in a fraction of human breast tumors [2], suggesting that additional mechanisms might be involved in functional inactivation of the p53 checkpoint in tumors expressing wild type p53. Additionally, Seladin-1 (under DHCR24 alias) is also involved in cholesterol metabolism [3]. Cholesterol metabolism is known to be a contributing factor in cancer progression.

In order to determine if Seladin-1 is a TSG involved in breast cancer we propose to: 1) Identify possible Seladin-1 mutations in primary breast tumors. 2) Investigate the alterations of Seladin-1 expression in breast cancer cells. 3) Perform functional assays on tumor-specific Seladin-1 mutants.

BODY:

Task1. Identify possible Seladin-1 mutations in primary breast tumors (months 1-12).

Initially, we will amplified the Seladin-1 exons from 60 breast tumor genomic DNA samples (all were obtained from a commercial source (Oncomatrix, Inc.), followed by the sequence analysis of the open reading frames. As a result, we did not we encounter finding mutations in the coding region and exon-intron junctions (other than known single nucleotide polymorphisms (SNPs).

However, we found high frequency (12-25%) new insertion/deletion polymorphism in the area close to exon7 (Fig.1). We observed a single nucleotide deletion (IVS6: delT -29...-36) in a stretch of seven consequent T upstream of exon 7 of Seladin-1 (see Fig.1) in genomic DNA of patients with breast tumors. This type of sequence belongs to a structure commonly referred to as the polypyrimidine tract. The exact size of polypyrimidine tract is gene-specific, but it has been shown that a single nucleotide deletion in this tract results in exon skipping or abnormal splicing, sometimes causing nonsense-mediated mRNA decay and resulting in several different inherited diseases.

Figure 1. In Pink: forward and reverse primer (remember that actual sequence is the reverse complement of what is shown for reverse primer only). Blue underlined is exon 7. Italics: a potential lariat branchpoint sequence. Stretch of T's with one nucleotide deleted (IVS6: delT –29...-36) in mutant is underlined.

Fr7fwd2 ➔ In mutant: TTTTTT

GCTTCTGTCAAGCACAGAATGGGACCTGGCCCCCTGGCCCCTTGCACTTGGAGACATTCAACCCTTGTTGGTTTTTTTTT

Exon7

CTCTCCAACCCCTAATCTTTGTTTTCAGGACATTATCCCCCTTGGCAACAACCCCATCTTCCGCTACCTCTTTGGCTGGATGGTGCTC
 CCAAGATCTCCCTCCTGAAGCTGACCCAGGGTGAGACCCTGCGCAAGCTGTACGAGCAGCACCAC
 GTGGTGCAAGACATGCTGGTGCCCATGAAGTCCTGCAGCAGGCCCTGCACACCTTCCAAAACGACATCCACGTGAG

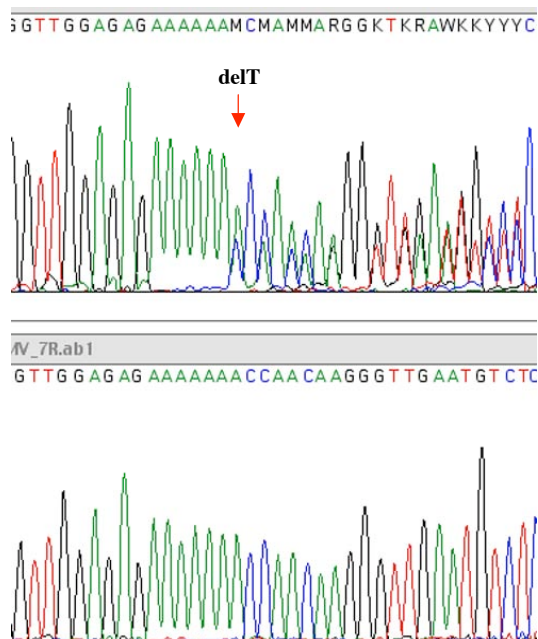
Fr7rev2 ←

TGGGCGAGGGCAGGGAGGGCGCACAGGTGCATTCCCTTGAATCCATGGTCTCCTCCCCTGAACCGTGTGACCGACCTTGGGCACACT
 GCCCTCTGGGTGTTTTCCCAACTTTAAGATGAGGTCCTTGTCCTATGTCTGCTGAGGCCTCAAC

The actual sequence analysis of one breast tumor sample with this polymorphism is shown in Fig. 2.

Figure 2. Mutation in human Seladin-1 intron-exon junction sequence (IVS6: delT –29...-36). Top-genomic DNA from primary human tumor (Reverse sequence shown), bottom-normal human DNA. Position of one nucleotide deletion (delT) is shown. Note that reverse complementary sequence is shown as indicated.

Reverse sequence



We analyzed whether the matching DNA from unaffected organs of the same patient. In case of this particular polymorphism, both tumor material and patients DNA obtained from unaffected tissues have the same polymorphism. However, we are now investigating whether this particular polymorphism is present with higher or lower frequency in breast cancer patient population comparing with unaffected individuals. If a statistically significant difference will be shown, this polymorphism might be important for the eventual development of breast cancer.

We submitted an abstract describing early stages of this research to Cold Spring Harbor Symposium on Quantitative Biology (see attached).

Task2. Investigate alterations of Seladin-1 expression in breast cancer cells (months 1-12).

Accordingly, we isolated RNAs from human breast cell lines MCF7, BTK20, T47D, HBL100, MDA-MB-231, MDA-MB-468 and MCF10A. Total RNA isolation was done with Trizol (Invitrogen) as described by the manufacturer. Seladin-1 Probe labeling and hybridization was done as described previously using commercial kits. As a control, we used RNA from three different batches of normal mammary epithelial cells (HMEC). For immunoblotting, we used antibodies against C-terminal domain of Seladin-1, described in [1].

When RNAs from 10 primary breast cancer cell lines were analyzed, the conclusions on Seladin-1 gene expression was that this gene is usually expressed at high level in breast cancer cells (with the exception of MDA-MB-231). Interestingly, expression of Seladin-1 in MDA-MB-231 cell line was subjected to regulation by 5aza-2'-deoxycytidine (5AzaDC), a demethylating agent. An increase in Seladin-1 expression in response to 5AzaDC indicates that this putative cell line was a candidate for promoter methylation studies using methylation-specific PCR and bisulfite sequencing. However, despite our best efforts, none of the primers designed to perform methylation studies had worked on promoter region of Seladin-1 (probably due to very high C/G content in those areas). More efforts in this regard are currently ongoing.

Task3. Perform functional assays on tumor-specific Seladin-1 mutants (months 7-12).

So far, we did not detect breast tumor-specific Seladin-1 mutants (in the coding region) and, therefore, we concentrated on mutations that we [1] and others [3] have previously described. Interestingly, we found that Seladin-1 had inhibitory effect on growth of some breast cancer cells (notably MDA-MB-231) expressing it at the low levels, while having no affect on the others: T47D or MDA-MB-468.

KEY RESEARCH ACCOMPLISHMENTS:

1. From analysis of 60 genomic DNA samples from patients with breast cancers, we did not find mutations in coding (exon) sequences, suggesting that a potential Seladin-1 mutation in breast cancer is a rare event.
2. We identified novel insertion/deletion polymorphism in a close proximity to exon 7 in Seladin-1 genomic sequence. We plan to analyze frequency of this polymorphism with relation to breast cancer and other malignancies.
3. Some breast cancer cell lines growth (MDA-MB-231) is inhibited by Seladin-1 in vitro.

REPORTABLE OUTCOMES:

1. ABSTRACT presented at the 70th Cold Spring Harbor Symposium on “Molecular approaches to controlling cancer”, 2005.
2. NIH RO1-A1 grant “Seladin-1 in RAS induced senescence and tumorigenesis” applied for in March of 2006.

CONCLUSIONS:

1. Seladin-1 is rarely mutated in breast cancer.
2. A novel high frequency (12-25%) Seladin-1 insertion/deletion polymorphism was found.
3. Seladin-1 inhibits growth of some breast cancer cell lines in vitro.

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SELADIN-1 MUTATION ASSOCIATED WITH MULTIPLE HUMAN TUMORS

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Expression of multiple oncogenes and inactivation of tumor suppressors is required to transform primary mammalian cells into cancer cells. Activated Ha-RasV12 (Ras) is usually associated with cancer, but it also produces paradoxical premature senescence in primary cells by inducing reactive oxygen species followed by accumulation of tumor suppressors p53 and p16^{INK4a}. We identified, using a direct genetic screen, *Seladin-1* as a key mediator of Ras-induced senescence (Wu *et al.* 2004, *Nature* 432, 640). Following oncogenic and oxidative stress, Seladin-1 binds p53 amino terminus and displaces E3 ubiquitin ligase Mdm2 from p53, thus resulting in p53 accumulation. This suggested that *Seladin-1* is a potential human tumor suppressor.

In order to test this hypothesis, we performed the sequence analysis of coding exons of Seladin-1 in 97 human sporadic cancers of various types. As the result, we observed that approximately 21% of all tested patients (20 out of 97) had the mutation at the single common site, suggesting that *Seladin-1* function is frequently affected in patients with multiple major tumor types. No known SNPs exist at this location and those were not detected at this site in 64 randomly chosen unaffected donors. The cancer mutation data and the significance of this mutation for Seladin-1 function will be presented.

The remarkably high frequency of the *Seladin-1* single-site mutation in patients developing multiple human tumors suggests a potentially unique requirement for, or a mechanism of, *Seladin-1* mutation in human cancers in comparison with the previously described tumor suppressors.