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11q21.1–11q23.3 is a Site of Intrinsic Genomic Instability Triggered by Irradiation

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Abstract

The chromosome location, 11q21–23, is linked to loss of heterozygosity (LOH) in multiple tumors including those of breast, lung and head and neck. To examine the process of LOH induction, the H292 cell line (human muco-epidermoid carcinoma) was irradiated or treated with anti-CD95 antibody, and individual clones isolated through two rounds of cloning. Regions of LOH were determined by screening a suite of eight polymorphic microsatellite markers covering 11p15–11q24 using fluorescent primers and genetic analyzer peak discrimination. LOH induction was observed extending through 11q21.1–11q23.3 in 6/49 of clones surviving 4 Gy and 8/50 after 8 Gy. Analysis of selected clones by Affymetrix 6.0 SNP arrays confirmed the initial assessment indicating a consistent 27.3–27.7 Mbp deletion in multiple clones. The telomeric border of LOH mapped to a 1 Mbp region of elevated recombination. Whole genome analysis of SNP data indicated that site-restricted LOH also occurred across multiple additional genomic locations. These data indicate that 11q21.1–11q23.3, and potentially other regions of this cell line are sites of intrinsic cell-specific instability leading to LOH after irradiation. Such deletions may subsequently be propagated by genetic selection and clonal expansion.

INTRODUCTION

Loss of heterozygosity (LOH) is an important factor in the pathogenesis of malignancy as it may stand as a marker location for involved tumor suppressor gene(s). Less is known of the process whereby LOH occurs during tumorigenesis, in particular whether LOH occurs entirely at random or is secondary to factors intrinsic to specific cells or cell types. LOH at 11q23 is observed in many different types of cancer, including 40% of all women with primary breast tumors, as well as those presenting with tumors of lung and cervix (Lee et al., 2000; Kuramochi et al., 2001; Honda et al., 2002; Nagahata et al., 2002; Martin et al., 2003; Tan et al., 2004; Zhang et al., 2005). Of several candidate genes detected within 11q23, *CADMI* (*TSLC1*, *IGSF4*) in particular has been localized to 11q23.2 and has been shown to be a tumor suppressor in lung, glioma, breast, cervix as well as the original definition in Non-small cell lung carcinoma (Kuramochi et al., 2001; Goto et al., 2005; Houshmandi et al., 2006). The same gene is also linked to silencing by promoter methylation (Allinen et al.,

2002). Other genes linked to carcinogenesis identified within this region of chromosome 11 include *MRE11A*, *ATM* and *H2AFX*, all of which are involved in DNA damage signaling or repair (Shiloh 2003). It is possible that more than a single gene may contribute to the transformation process within this region where LOH may extend over several Mbp (Lee et al., 2000; Tan et al., 2004; Zhang et al., 2005).

The contribution of LOH to the genesis and development of tumors is linked to the proliferative advantage provided to individual cells that lack key suppressor genes within the region of loss. Additionally, such a growth advantage requires suppression of the alternate allele through either mutation or epigenetic restriction such as methylation (Oldenburg et al., 2007). More recently, evidence has been gathered from the stroma surrounding developing tumors from the head and neck of smokers indicating that this also is subject to LOH (Weber et al., 2007). Within both the developing tumor and non-malignant stroma comparable aberrations were found, an observation also reported for breast tumors (Fukino et al., 2004; Weber et al., 2007). Although another study has not confirmed these findings of LOH within normal tissue (Qiu et al., 2008). If correct however, these data may reflect the long standing concept of field cancerization in which histologically abnormal tissue is observed at the perimeter of malignant disease (Slaughter et al., 1953; Braakhuis et al., 2003). In the modern era, this idea has developed a genetic rationale in that cells are produced, perhaps within a stem cell compartment, that undergo selective modification providing a pool or patch of similarly modified cells (Braakhuis et al., 2003). Interestingly, studies of such patches with TP53 as the focus find mutations at multiple codons, not completely in accord with simple clonal expansion, but perhaps representing co-existent multiple events (Waridel et al., 1997).

Currently, several alternative mechanisms have been proposed to describe the generation of LOH, including chromosomal deletion, mitotic nondisjunction, and recombination between homologous chromosomes (Lasko et al., 1991; Stark and Jasin 2003; Lai et al., 2007). The selective growth advantage derived from the loss of specific genes within affected regions may subsequently provide the reproducible deficiencies observed within the 11q23 region of tumors from many different sites (Lee et al., 2000; Nagahata et al., 2002; Martin et al., 2003; Tan et al., 2004; Zhang et al., 2005). An alternative, but not mutually exclusive hypothesis is the possibility that some parts of the genome are selectively prone to LOH and thus produce a cohort of cells with an enhanced capacity for growth. This idea is supported by the observation that fields of cells may carry similar, but not identical LOH defects in multiple cells, an observation unlikely to arise from the expansion of single or small numbers of cells containing different lesions (Fukino et al., 2004; Weber et al., 2007). We propose here that reproducible aberrations seen within 11q23 may not be simply due to clonal self-selection, but may be driven by both host cell factors and the intrinsic hypersensitivity of the 11q23 region.

In the work described we used a cell line, NCI-H292, derived from a lymph node metastasis of a pulmonary muco-epidermoid carcinoma, to study the induction of LOH by irradiation. This cell line was chosen in that it is derived from a class of tumors that are pre-disposed to aberrations at 11q, specifically, *MECT1-MAML2* translocations that deregulate CREB mediated signaling (Wu et al., 2005). In particular, this cell line both contains the signature fusion between chromosome 11 and 19 and two intact copies of chromosome 11 (Tonon et al., 2004). Multiple unique clonal derivatives of the primary tumor cell line were analyzed for LOH using polymorphic markers for chromosome 11, confirmed by both SNP arrays and FISH, using a whole chromosome 11 probe. In multiple clones, a common region of radiation-induced LOH was found at 11q21.1–11q23.3 that covered a near identical tract of DNA, extending over 27 Mbp. These data indicate that the changes seen are likely linked to

intrinsic genomic susceptibility at 11q23 and are not the result of LOH events initiated at random.

METHODS

Cell Line

The NCI-H292 cell line was obtained from the American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 (ATCC) supplemented with 10% FBS and 5% PSA antibiotic/mycotic solution (Gibco, Carlsbad, CA).

Cell Survival

Clonogenic cell survival assay following irradiation was executed by plating between $1-10 \times 10^3$ cells in triplicate and scoring colonies of 50 cells or more after approximately 14 days of growth. Cell cultures were also exposed to a range (0.25–4.0 $\mu\text{g/ml}$) of anti-CD95 antibody (eBioscience, San Diego, CA) and subjected to clonogenic analysis as for the irradiated samples.

Isolation of Individual Clones

In order to isolate individual clones, preliminary cell survival experiments were conducted to determine the appropriate cell numbers to generate single, discrete colonies. For control, 4 Gy and anti-CD95 antibody treatment, 1000 cells were seeded and for 8 Gy treatment 10,000 cells were seeded in 100 mm Petri dishes in triplicate. In this setting, the anti-CD95 antibody sample set was used as a control providing toxicity to the cells without the direct genomic damage induced by irradiation. The cells were either irradiated in complete media with 4 or 8 Gy in a Nordion Cs-137 irradiator system or exposed to 0.5 $\mu\text{g/ml}$ anti-CD95 antibody for 48h, washed free of the antibody and then fresh media was added. Immediately after treatment, cells were returned to culture until identifiable colonies were formed. At this stage the media was removed by aspiration and single colonies picked by careful addition of 1–2 μl of a 0.25% trypsin/EDTA solution to macroscopically observable colonies using more than one of the triplicate sample plates. Viable cells from single colonies were then released by gentle agitation and placed into 96 well plates containing 200 μl of a 50:50 mixture of fresh and conditioned (24 h in contact with viable H292 cells followed by filtration) medium and cultured until 50% confluence. Cells from each of the 96 wells used were subsequently recloned in 6 well plates to form identifiable colonies. These colonies were picked as before, expanded in culture, and then cells were either stored in liquid nitrogen or used to extract genomic DNA with Promega Wizard kit (Madison, WI). Approximately 50 separately identifiable colonies were isolated for each treatment.

Polymorphic Markers

Polymorphic marker sets covering the entire chromosome 11 were produced by inspection of the Marshfield and NCBI databases. Individual primer pairs were identified for each marker set to confirm heterozygosity. This step was essential for subsequent analysis of LOH. Marker sets that lacked heterozygosity were dropped from the study. Confirmation of heterozygosity and subsequent analysis used PCR executed through 30 cycles with an initial 5 min denaturation step at 95°C, subsequent steps were 94°C for 30s, followed by annealing between 50–60°C (depending on primer set) for 30s and extension at 72°C for 30s, with a final extension at 72°C for 5 min.

Polymorphic marker sets used are as follows:

<i>D11S1999</i>	F - 5' TACATGGCAGCAGGCATATA 3'
	R - 5' GAGTAAACAAGATTGCTAGATAGG C 3'
<i>D11S2011</i>	F - 5' CATCTGTCTGTCCATCTGTCC 3'
	R - 5' CAATCCTATTGCACTGGG C 3'
<i>D11S1366</i>	F - 5' GCTACAATGATAGGGAAATAATAGA 3'
	R - 5' GGTGGGATCCTTTGCTATTT 3'
<i>D11S2000</i>	F - 5' AGTAGAGAACAAAACACTGTGGC 3'
	R - 5' TTTGAAGATCTGTGAAATGTGC 3'
<i>D11S3178</i>	F - 5' CCACGCCTGGCTCATT 3'
	R - 5' GAGGATTTTCAGCAGCAGA 3'
<i>744724</i>	F - 5' AATGCTTGCATGGACTAGGG 3'
	R - 5' ACAGCAAGCAGCAAAAGT GA 3'
<i>D11S1340</i>	F - 5' GCTGAATGAGTCCTGAGTAATAA 3'
	R - 5' GGCCTAGACGTTCTTTGTG 3'
<i>D11S4464</i>	F - 5' ACATTTCCAAGGGCTACACA 3'
	R - 5' CCTCACTGAGTCATGTTGCA 3'

Analysis of each polymorphism was executed by substituting a single 6-FAM labeled primer in the PCR reaction and analyzing the data on an ABI 3730 XL capillary sequencer (Applied Biosystems, Foster City, CA). Data after analysis was further processed by “Peak Analyzer” software (Applied Biosystems).

Single Nucleotide Polymorphism Analysis

Single nucleotide polymorphism (SNP) analysis was executed using the Genome-Wide Human SNP Array 6.0 (Affymetrix, Santa Clara, CA). Genomic DNA was isolated from fresh cells grown from previously isolated clones using the manufacturer's provided protocol. Genomic DNA was hybridized according to manufacturer's protocol on the Affymetrix Genome-Wide Human SNP Array 6.0 which features 1.8 million genetic markers, including more than 906,600 single nucleotide polymorphisms (SNPs) and more than 946,000 probes for the detection of copy number variation. The arrays were washed and stained on a Fluidics Station 450 and were scanned on a GeneChip Scanner 3000. The manufacturers' Genotyping Console software was used to analyze LOH.

FISH Analysis of Chromosome 11

2.5×10^5 H292 cells were plated in 6 well plates (Thermo Fisher Scientific, Waltham, MA) 24 hours prior to treatment with either no irradiation, 2Gy or 4Gy and subsequently incubated at 37°C, 5% CO₂. After 72 h, 10 µl of colcemid (10 µg/ml solution, Gibco) was added to each well and incubated for 30 min at 37°C. Cells were then lysed with hypotonic KCl (0.075 M) and fixed in three changes of 3:1 methanol:acetic acid and dropped onto slides. Subsequently the chromosome spreads were denatured for 2 min at 65°C in 70% de-ionized formamide (Applied Biosystems), 0.6xSSC (saline-sodium citrate buffer) and dehydrated by washing with increasing concentrations of ethanol. FITC labeled whole chromosome 11 paint probe (Genetix, Boston, MA) was denatured for 10 min at 65°C, followed by 30 min at 37°C. Ten µl of the probe was applied to each slide, covered with a cover slip and sealed with rubber cement. Hybridization was performed in the dark at 30°C overnight. Post hybridization processing consisted of two 50% formamide, 0.5x SSC

washes, two 1xSSC washes and two 4xSSC, 0.05% NP-40 washes for 5 min at 45°C each. Chromosomes were counterstained with DAPI mounting medium (Santa Cruz Biotech, Santa Cruz, CA), covered with a cover slip and viewed under a Leica DMR fluorescent microscope. Photographs were taken with a Leica DC 300F (Leica, Heerbrugg, Switzerland) camera and processed with Image-Pro Plus 5.1 software (Media Cybernetics Inc., Bethesda, MD).

Database Sources

Using the UCSC genome browser it is possible to rapidly screen multiple structural and functional motifs throughout the genome. With this tool a screen was conducted for regions of enhanced recombination (Kong et al., 2002). The deCODE database that contains the most comprehensive data set in terms of sample size was used for estimating recombination frequency within chromosome 11. Similarly an estimate of deletion polymorphisms was also undertaken using a database initially created as a part of the HapMap Project (Kegel et al., 2007).

RESULTS

Clonal Analysis

Following the protocol described, 51 control H292 clones were available for analysis and 49, 50 and 46 following 4 Gy, 8 Gy and anti-CD95 antibody treatments respectively. Assessment of the surviving percentage using a clonogenic assay indicated that approximately 25% of cells survived 4 Gy and 6% survived 8 Gy. After exposure to the 0.25–4.0 µg/ml of anti-CD95 antibody a plateau in toxicity was reached at 0.5 µg/ml with approximately 1% survival. This concentration was subsequently used for the generation of clonal survivors.

LOH Determined by Polymorphic Marker Analysis of Microsatellite DNA

LOH analysis was performed on all isolated clones. In this study only a complete loss of one marker was used as a recordable event, all such events detected are shown in Table 1 and a sample response in Figure 1. Other studies using biopsy or similar material require the use of less rigorous criteria of allele suppression to take account of possible contamination with normal heterozygous cells (Fukino et al., 2004). The two rounds of cloning used here however permits absolute determination of LOH in each unique clone. Variations in peak intensity of each allele were sometimes noted but this is more likely a PCR related phenomenon than any biological alteration at the target site.

Analysis of all polymorphic microsatellite markers used showed a total of 14 clones that had evidence of LOH, 6 after 4 Gy and 8 after 8 Gy. Using Fishers exact test the induction of LOH by irradiation was significant ($P=0.027$ at 4 Gy and $P=0.0067$ after 8 Gy). No LOH in chromosome 11 was observed in any control clone or those exposed to the pro-apoptotic agent anti-CD95 antibody. LOH events were observed over a discrete region spanning 11q21.1–11q24.1 (Table 1). A sample of the results obtained is shown in Figure 1 for a single clone and marker set *D11S2000*. No events were observed more centromeric than 11q21, or in the 11p arm. Across all doses, 10 individual clones were observed that contained losses of the same 5 contiguous microsatellite markers covering a range of 11q21.1 (*D11S1366*) to 11q23.3 (*D11S1340*), a distance of 19.69 Mbp (Figure 2). Two additional clones had contiguous loss covering four markers (Figure 2). In no instance did microsatellite loss alternate with a marker that retained heterozygosity, suggesting that the contiguous LOH observed was initiated from a single event. Of specific note, all microsatellite losses except two (at 11q24) were homologue specific, losing material linked to the larger PCR marker product only (see comment below).

SNP Analysis

Using the latest version of Affymetrix SNP 6.0 Chip, complete genome LOH screening may be carried out using the precision of single events with a median separation of ~700bp. With this level of precision a selection of clones were studied as representative of the data already gathered using microsatellite polymorphic markers (Figure 3 and Table 2). Immediately apparent was the confirmation of the extensive LOH tract loss observed using the microsatellite polymorphic markers. With the increased precision offered by the SNP analysis it was found that the extent of loss was highly reproducible, 4 of the clones analyzed were found to have very similar levels of LOH of 27,285,040 to 27,684,400 bp (hereafter called large tract LOH) with most variation occurring at the centromeric border. All large tract LOH was also linked to a reduction in copy number, marking these events as interstitial deletions. Such reproducibility was unexpected and raises the question of possible clonal contamination. This is however viewed to be extremely unlikely in that the clones were picked after two rounds of single cell cloning, individual clones were picked from separate original culture plates and the clones assessed include cells from two completely different experimental cell populations (4 or 8 Gy). Thus it is concluded that these repetitive LOH losses are not the result of cloning the same population.

It was also noted that in 4 irradiated and 1 control sample, that lacked large tract LOH, each exhibited a discrete, approximately 1 Mb region of LOH at the telomeric border of the large LOH tract in 11q. A control sample was also observed that lacked both large tract LOH and this, smaller, 1 Mbp tract (Figure 3). This suggested that this restricted region of LOH was derived from events that occurred during the experiment and were not pre-existent in the parental cell line. Analysis of Clone 18 (8 Gy) showed marker *D11S4464* as positive in Figure 2 that using SNP analysis corresponded with the ~1Mbp LOH, the detailed SNP analysis showing increased telomeric extension of LOH that now includes the *D11S4464* microsatellite marker. Interestingly, this specific marker, now linked to the presence of short tract LOH, was the only microsatellite to show LOH involving the alternate homologue. As mentioned above, all LOH involving microsatellite markers involved a single homologue, excepting those at *D11S4464*.

Further analysis of this particular 1 Mb LOH tract was undertaken using the UCSC Genome browser tool, screening for sequence repeats and other variations (Kuhn et al., 2009). Using this approach it was found that the 1 Mb of LOH within 11q24 co-mapped to a 1 Mb high frequency (3.4 cM/Mb) recombination tract. This was the joint second highest recombination rate, measured per Mb, for all of chromosome 11 using the deCODE dataset containing 5,136 microsatellite markers for 146 families with a total of 1,257 meiotic events (Kong et al., 2002). This implies that this specific region of the genome is intrinsically unstable, a finding supported by the spontaneous generation of LOH at this location both with and without irradiation. A similar screen of the centromeric border of the long tract LOH was undertaken with the same goal of identifying a possible explanation for the sharp cutoff observed here. Of all the elements screened, a deletion map undertaken as a part of the HapMap project studying 30 individuals of European (CEU) and 30 of African origin (YRI) showed a deleted region in 3/30 of the YRI group at location 95,443,728–95,466,766bp, immediately (10 kbp) adjacent and centromeric to 2/4 of the large tract LOH (Table 2). This deletion was approximately 14Mbp distant from any other deletion from either CEU or YRI groups, marking this area as potentially genomically unstable (Conrad et al., 2006).

LOH and Irradiation

Within the approximately 200 individual clones analyzed, only those that were irradiated with either 4 or 8 Gy showed large tract LOH within chromosome 11. However the smaller

LOH element at the larger tract telomeric border was seen in both irradiated and un-irradiated clones. Thus the radiation itself has a clear and essential role in inducing at least the large tract region of LOH, in accord with other published work (Moynahan and Jasin 1997). Also clear from a screen of LOH throughout the genome within the sample set analyzed here, made possible by the SNP array technique, is that radiation alone is insufficient to induce LOH at the high frequencies observed here. This is an important point in that irradiation will deposit energy at random, potentially generating broadly distributed LOH throughout the genome. This is however unlikely to be the case, as analysis of chromosomes 12 and 13, that are of comparable size to chromosome 11 (and therefore a similar target for radiation induced DNA breaks and damage), show no comparable LOH in any of the 10 clones examined using SNP's. However, a screen of all samples analyzed did reveal LOH throughout the genome that was seen in both irradiated samples and, to a lesser extent, in those that were left untreated. These data, shown as Figure 4, demonstrate that though chromosome 11 appears to be the most sensitive to the process of LOH in this system, other chromosomes also participate. Of note, most regions of LOH observed co-locate to the same genomic position within different clones. Thus the region-restriction observed in detail at chromosome 11q appears to be a common finding throughout the genome of these multiple clones. Though the commonality observed is clear on the macroscale, at the molecular scale there is still variation. For example, the four regions noted at 5p15.33 contain three of their centromeric borders clustered closely together and one that is 100 kbp distant. This is similar to the variability at the borders of LOH within 11q and shown in Table 2. These data therefore suggest that the location of LOH is likely influenced by local factors related to DNA sequence or chromatin structure. This is compelling evidence that LOH induction at these high frequencies by irradiation is the result of both DNA damage, and enhanced susceptibility to such damage at discrete genomic locations.

FISH Analysis

Though both the polymorphic marker analysis and SNP arrays provide a detailed description of LOH, visual verification of the process was sought using FISH technology (Figure 5). Here, whole chromosome painting of chromosome 11 was utilized to obtain an image of each chromosome 11 in addition to the duplicated and translocated copy. In Figure 5A the control H292 FISH image shows both intact chromosomes. In Fig 6B, an image of each chromosome is seen where one is shortened, such a shortened copy of chromosome 11 was only observed in the irradiated samples of H292. The loss of ~27 Mbp of material from chromosome 11 will result in ~20% decrease in absolute size of this chromosome, a difference observable using light microscopy. These data provide visual evidence supporting the generation of LOH indicated using the molecular techniques discussed above. On both images additional fluorescence can be observed corresponding to the 11,19 reciprocal and 11,13 non reciprocal translocation that are associated with this cell line (Tonon et al., 2004).

Microsatellite Instability (MSI)

From the distribution analysis of LOH events it is apparent that the 11q21.1–11q23.3 region is particularly susceptible to radiation-induced effects. Though the reasons underlying this are likely multi-factorial in nature, further analysis of this region showed additional aberrations within the clonal survivors receiving either no treatment, or after exposure to anti-CD95 antibody (Figure 6). The anti-CD95 antibody exposure was used here as a cytotoxic control with no known genotoxic potential. As discussed, neither of these groups showed any evidence of LOH at 11q21.1–11q23.3 as documented by losses in the markers used. At a single marker location however (11q23.3 – Marker set 744724), evidence of microsatellite instability was observed. This particular polymorphic microsatellite marker was unusual in that each allele exhibited a double peak, observed in both MCF10 (data not

shown) and H292. In this case 4/51 (7.8%) control clones and 7/46 (15.2%) anti-CD95 antibody treated clones showed evidence of MSI as either a size shift in one marker compared to its unmodified form or the addition of a separate peak retaining the original peak profile as well. No irradiated clone exhibited any MSI at any location. Comparing the total number of irradiated clones that exhibited MSI (0/99) with those showing MSI that were not irradiated or received anti-CD95 antibody treatment (11/97) these observations were significant at the $P=0.0008$ level (Fishers exact test). It appears that in all cases both the new or additional peaks were equivalent in size to the loss of a single triplet to the smaller of the two markers and, in contrast with the studies on LOH, all alterations were restricted to the same (smaller) polymorphic marker allele, indicating homologue restricted instability to the alternate parental chromosome set. This suggests that the location of the 744724 marker in this cell line was particularly susceptible to replication errors and defines the instability phenotype as MSI-Low (minority of affected markers showing effect) (Boland et al., 1998).

DISCUSSION

The presence of LOH in human tumors is of clear significance in defining the likely location of tumor suppressor genes (Martin et al., 2003; Fukino et al., 2004; Tan et al., 2004; Zhang et al., 2005; Weber et al., 2007). The generation of discrete regions of LOH, other than through whole chromosome deletion may be classified into five general groups based on chromosome analysis (Ogiwara et al., 2008). These partial (retaining the specific chromosome) LOH events are mitotic recombination, mitotic gene conversion, terminal deletion, interstitial deletion, and an unbalanced translocation. As in the cases reported here all large tract LOH was associated with copy number change, thus the process of LOH observed was a true interstitial deletion. This is in contrast to a possible copy number neutral event where either a complete or partial chromosome 11 loss occurred followed by duplication of the remaining copy. In these studies, 6/49 H292 clones surviving 4 Gy and 8/50 surviving 8 Gy exhibited LOH, extending in most (10/14) cases over long tracts of DNA in excess of 27 Mbp. No instances of such large scale LOH were observed in the 51 control clones or the 46 treated with anti-CD95 antibody. Additionally, no LOH was observed within a group of MCF-10 breast cancer clones that were similarly isolated after exposure to both 4 and 8 Gy (data not shown). In a similar study to this one, Boyd et al. found 8 microsatellite defects in a human glioma cell line after analysis of 100 clonal survivors of a 3 Gy radiation exposure (Boyd et al., 2000). Others have shown that LOH is strongly stimulated by a DNA double strand break however in these experimental systems the frequency of LOH was much smaller (Stark and Jasin 2003). These latter studies also used engineered reporter substrates which might not faithfully duplicate local chromatin architecture, or widespread epigenetic features such as methylation that cover Mbp sized regions. In addition, such studies do not reproduce the extensive loss of DNA sequence which is observed both clinically and in the data reported here. One additional difference between our data and those of others is that we studied a region of documented LOH significance, 11q23, as determined by screens of human tumor material (Martin et al., 2003; Tan et al., 2004; Zhang et al., 2005).

An unexpected finding of these experiments was the induction of homologue specific LOH. It might be expected that if LOH were truly a random phenomenon, then each allele would be equally at risk. Here an almost complete bias towards the loss of the larger polymorphic marker was observed. In studies of LOH using patient samples, no such bias is likely to be observed as every genome is derived from a unique parental source, masking any inherent bias in individual samples. In this study, multiple clones were isolated from a single cell line providing a common background. Others have demonstrated this effect in mouse crosses whereby specific chromosomes preferentially undergo LOH at one homologue. In particular,

chromosome 2 within the F1 cross from CBA/H - C57BL/6 exhibits homologue restricted LOH after irradiation (Rigat et al., 2001). The parallel here is that such animals are isogeneic and are thus similar to the clonal cell line derivatives studied here. Though the explanation for this homologue restriction is unclear, it does suggest that LOH, at least in this model, is a non random process.

In order to provide confirmation of the LOH detected by microsatellite polymorphism analysis, a subset of both LOH positive and negative clones were analyzed by SNP arrays. Here, the presence or absence of individual SNP's may be used as a marker for LOH over the entire genome, both extending the range of this study and the level of precision offered. Using this technique on a subset of the clones showed complete concordance with the large tract LOH found using the microsatellite polymorphic marker set. In addition, the boundaries of the LOH tract determined in separate clones were very similar, in some cases at the level of precision provided by the technique of individual SNP's (Figure 3 and Table 2). This near equivalence of individual clones in terms of LOH deletion size was unexpected and prompted a closer analysis of the region involved. As observed in Figure 3, analysis of SNP data in clones that lacked the large LOH region, a discrete ~1Mbp region of LOH was found in some control and irradiated samples at a position closely matching the telomeric border of the larger LOH tract. The fact that a control sample lacked this specific LOH region suggests that LOH here arose during the process of the experiment, indicating a region of genomic instability.

To gain an independent assessment of the potential instability of this location, the UCSC genome browser was utilized to screen the involved region for potential structural or functional deficits (Kuhn et al., 2009). With this system it is possible to screen for a large number of elements such as CpG islands, nucleosome occupancy, repetitive DNA such as SINES or LINES and many others. Using the human linkage map databases accessible within this program it is possible to assign a recombination score to discrete regions of the genome, here scored on a per/Mbp basis (Kong et al., 2002). Using the deCODE database that contains the most comprehensive data set in terms of sample size, it was found that the location with the second highest recombination frequency for all of chromosome 11 overlapped the single Mbp region of LOH (Figure 3). This suggests that this particular region is sufficiently unstable to facilitate LOH but it does not support LOH beyond a restricted 1Mbp tract. Only those cells exposed to irradiation, an agent inducing DNA double strand breaks, were able to generate the long tract LOH observed.

In this context it may be significant that of all microsatellite markers analyzed, only two, at *D11S4464*, were observed to involve the alternate homologue (Table 1 and Figure 2). It is now apparent that at least in the LOH in Clone 18 (8 Gy) where both microsatellite markers and SNP arrays were used, this region maps to the same 1 Mbp region of instability. It is proposed that the initial LOH instability (short tract LOH) that is observed, in conjunction with radiation damage of the alternate homologue, may result in a failed attempt at recombination mediated repair between homologues. Therefore it is likely that the recombination instability observed in addition to the DNA damage from the irradiation contributed to the long tract LOH observed. A simple calculation, based on 30–35 DNA double strand break per Gy per diploid cell, shows that after 4 Gy, the distribution of recombinogenic lesions, such as DNA double strand breaks, occurs at a frequency of 1 break approximately every 25 Mbp (Kegel et al., 2007). Thus it is likely that the majority of cells irradiated with this dose will have received at least one DNA double strand break within the large tract region of LOH detected. Others have shown radiation is a potent inducer of genomic instability (Kimmel et al., 2008).

Using a similar approach, the centromeric border of the long tract LOH was analyzed for the presence of motifs that might contribute to its sharp termination. The region was adjacent (10 kbp) to a deletion polymorphism site suggesting a location of genomic instability (Conrad et al., 2006). Interestingly, the deletion site detected was mapped by the authors to include the *MAML2* gene that is frequently observed as a fusion gene partner (Fehr et al., 2009). These fusions are observed in a range of tumors, of mostly mucoepidermoid carcinoma type, the same pathology as the cell line used in this study and, significantly, the cell line used here does contain a copy of the *MECT1-MAML2* fusion gene as well as two normal copies of chromosome 11 (Tonon et al., 2004). In addition, *MAML2* in leukemia is found fused to *MLL* (Bell et al., 2008; Achcar Rde et al., 2009; Fehr et al., 2009). In this latter case, the fusion occurs within a gene located in the telomeric portion of the large tract LOH region of 11q discussed here. Two interpretations of the data presented here are proposed in relation to the presence of the intrinsic *MECT1-MAML2* fusion gene in the H292 cell line. First, the disruption to the normal genomic sequence at 11q21.1 through the *MECT1-MAML2* fusion may perturb attempts at homologous recombination repair of DNA damage in this region. This is however not considered likely as other sites of similar repetitive LOH are seen at other genomic locations within the small group of clones assessed by SNP array (Figure 4). Alternatively, the presence of this specific fusion gene itself may, through its affect on normal cell biology, globally affect the ability to suppress LOH. The *MECT1-MAML2* fusion gene is known to bind CREB, recruit p300/CBP and constitutively activate CREB-dependent transcription (Wu et al., 2005). Thus this fusion gene replicates constitutive activation of cAMP signaling targets by activating CREB directly and is a transforming event in cell based assays. This potentially links the CREB pathway with both the potent facilitation of LOH induction and the oncogenic process itself. The observations we have made are clearly cell-type specific as repetition of the experiments described here using MCF-10 cells showed no 11q LOH induction (data not shown). In terms of the mucoepidermoid histology of the H292 cell line used, others have noted that cells of this type are prone to repetitive aberrations within both 11q23 and other sites within the genome (Tonon et al., 2004). In this work we show that this characteristic is retained in terms of radiation-induced aberrations, raising the question of whether such site-specificity is unique to this cell type or may be a more widespread phenomenon.

Though it is possible to propose correlations of these data with that extracted from database resources, more direct information was sought from the data collected in the present study. It was noted that 7.8 % of the controls and 15.2 % of clonal survivors after anti-CD95 antibody treatment showed indications of microsatellite instability (MSI) for a total of 11/97 clones. Here, the heterozygous distributions of polymorphic microsatellite markers were modified by the appearance of new, smaller, PCR products using the 744724 marker set (Figure 6). The change corresponded to a single triplet loss. All clonal survivors had undergone two rounds of single cell cloning comprising ~20 generations each time. This suggests that in these clones the rearrangement had arisen during clonal expansion, presumably directly related to errors in replication linked to the mismatch repair (MMR) pathway (Edelbrock et al., 2009). No other microsatellite marker analyzed showed any similar alteration in marker size. The potential for repeat alteration has been linked to chromatin context such as location within a CpG island (Brock et al., 1999; Kovtun and McMurray 2008). Examination of the region surrounding 744724 using the UCSC tool showed no CpG islands in the immediate vicinity. Of interest, none of the clones assessed after irradiation (0/99) showed any evidence of MSI. Thus radiation exposure suppressed MSI development during the experiment, suggesting irradiation interfered with the MSI phenotype, possibly related to the recruitment of MMR proteins to sites of DNA damage (Hong et al., 2008). It is not known, however, whether the converse may be true, that is whether radiation-linked recruitment of MMR proteins is linked to the generation of LOH. As one possible association, it has been shown that MMR function is primarily executed at

the replication fork, a location where DNA synthesis is rapidly suppressed following irradiation, a p53 dependent process linked to PCNA phosphorylation (Shimura et al., 2006). One potential explanation that may link both defects in MMR and the LOH induction observed is the over-expression of the *MECT1-MAML2* fusion gene. This fusion gene is known to specifically deregulate CREB-linked signaling pathways that are implicated in multiple cellular process including cell cycle control and repair (Mohan et al.). In the latter case the CREB CBP axis is involved in regulation of base excision repair mediated by Thymidine DNA glycosylase, over-expression of the *MECT1-MAML2* fusion gene has the potential to disrupt this, as well as multiple other processes. Additionally, in colon cancer typified by microsatellite instability there appears an obligate requirement for mutation of both CBP and the highly homologous p300 protein that are deregulated in the cell line studied here (Ionov et al., 2004). Interestingly, the p300 protein is known to acylate p53 in response to DNA damage, suggesting yet another way in which DNA damage responses are likely altered in this cell system (Grossman 2001). Therefore it is possible the MSI observed is secondary to cellular perturbation linked to the *MECT1-MAML2* fusion itself.

In terms of a possible impact on the treatment of tumors with irradiation, it has been reported that cell lines lacking large regions of 11q, including the region discussed here, are both more genomically unstable and exhibit increased radiation resistance (Parikh et al., 2007). The deleted region contains at least three key genes involved in damage recognition and repair after irradiation, *MRE11*, *ATM*, and *H2AFX*. Loss of these genes is more commonly linked to increased radiation sensitivity suggesting that the 11q loss phenotype is more complex than simple deletion of specific genes (Thompson and Schild 2002). The lack of this region however, in combination with defects in other repair systems such as MMR, may explain the increased radiation resistance observed. Selective deletion of this region during the course of irradiation may therefore promote a more resistant phenotype in irradiated survivors.

The data we present here shows that the induction of LOH we observe in the mucoepidermoid H292 cell line is a non-random process that may be associated with the disruption of CREB dependent cellular signaling. If so, this presents an additional avenue for the study of LOH in what has classically been considered a stochastic or random accumulation of DNA loss.

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Figure 1. Polymorphic marker analysis for *DIIS2000*

Figure 2. LOH determined by microsatellite polymorphic marker analysis

Figure 3. LOH determined by SNP analysis of specific clones

Clone# Location	0 Gy	4 Gy						8 Gy		
	18 41	3	7	30	35	49	50	18	27	
2A (p22.3)										
2B (q24.2)										
4 (q28.3-q35.2)										
5 (p15.33)										
6A (q16.3-q22.1)										
6B (q25.2-q27)										
8 (p23.3-q13.1, 13.2)										
9 (q12-34.3)										
10 (p14)										
11A (q21-24.1)										
11B (q24.1)										
11C (q25)										
13A (q13.3-34)										
13B (q31.2-31.3)										
15 (q24.1-26.3)										
17										
18A (p11.32-11.21)										
18B (q12.2-12.3)										
19A (p13.11-12)										
19B (p13.2)										
XA (p11.22)										
XB (q23)										
Avg LOH/clone	2	5						6.4		

Figure 4. Global SNP analysis of LOH in control and irradiated clones

Figure 5. FISH analysis of Chromosome 11

Figure 6. Trace from polymorphic microsatellite marker analysis of 744724

Table 1

Number of clones with LOH at each polymorphic microsatellite marker location

Marker	Location	Radiation Treatment		
		0 Gy ^a	4 Gy	8 Gy
<i>D11S1999</i>	11p15.3	0	0	0
<i>D11S2011</i>	11q13.5	0	0	0
<i>D11S1366</i>	11q21.1	0	6	4
<i>D11S2000</i>	11q22.3	0	6	6
<i>D11S3178</i>	11q23.2	0	6	6
<i>744724</i>	11q23.3	0	6	6
<i>D11S1340</i>	11q23.3	0	6	6
<i>D11S4464</i>	11q24.1	0	0	2

Gy^a 1Gy is a unit of radiation absorbed dose equivalent to 1J/Kg

Table 2
Correlation between polymorphic marker analysis of LOH with SNP array based technique.

Clone	Dose (Gy)	Polymorphic marker analysis ^a	SNP analysis		
			SNP analysis ^a	Centromeric border	Telomeric border
18	0	X	X	121,742,580	122,761,720
41	0	X	X	null	null
03	4	✓	✓	95,077,380	122,761,780
07	4	X	X	121,709,280	122,817,940
30	4	X	X	121,763,680	122,779,840
49	4	✓	✓	95,476,720	122,761,760
35	4	✓	✓	95,476,040	122,761,760
18	8	X	X	121,763,690	122,913,380
27	8	✓	✓	95,345,780	122,761,760
50	8	X	X	121,708,780	122,761,760

Correlation between polymorphic marker analysis of LOH with SNP array based technique. 100% correlation between techniques in terms of large tract LOH, greater precision of SNP based analysis revealed more information on the borders of each LOH tract.

^aIdentification of large tract LOH ✓ - Yes; X - No