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RESEARCH ARTICLE

Nonreciprocal Chromosomal Translocations in Renal Cancer Involve Multiple DSBs and NHEJ Associated with Breakpoint Inversion but not Necessarily with Transcription

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Chromosomal translocations and other abnormalities are central to the initiation of cancer in all cell types. Understanding the mechanism is therefore important to evaluate the evolution of cancer from the cancer initiating events to overt disease. Recent work has concentrated on model systems to develop an understanding of the molecular mechanisms of translocations but naturally occurring events are more ideal case studies since biological selection is absent from model systems. In solid tumours, nonreciprocal translocations are most commonly found, and accordingly we have investigated the recurrent nonreciprocal t(3;5) chromosomal translocations in renal carcinoma to better understand the mechanism of these naturally occurring translocations in cancer. Unexpectedly, the junctions of these translocations can be associated with site-specific, intrachromosomal inversion involving at least two double strand breaks (DSB) in *cis* and rejoining by nonhomologous end joining or micro-homology end joining. However, these translocations are not necessarily associated with transcribed regions questioning accessibility per se in controlling these events. In addition, intrachromosomal deletions also occur. We conclude these naturally occurring, nonreciprocal t(3;5) chromosomal translocations occur after complex and multiple unresolved intrachromosomal DSBs leading to aberrant joining with concurrent interstitial inversion and that clonal selection of cells is the critical element in cancer development emerging from a plethora of DSBs that may not always be pathogenic.

INTRODUCTION

Chromosomal translocations are frequently associated with cancer development by interfering with genes at their junctions, most often by gene fusion (Rabbitts, 2009). In some cases, interstitial deletion and other rearrangements also cause gene activation such as SIL-TAL1/SCL deletion (Aplan et al., 1990) or give rise to gene fusions, such as in prostate cancer (Tomlins et al., 2005). This type of abnormal, somatically created chromosome is common in a spectrum of human cancers (Rabbitts, 2009). The mechanisms that underlie the creation of these aberrant chromosomes are poorly understood except in cases, generally restricted to lymphoid tumors, where the Recombination Activation Gene recombinase machinery mistakenly recognises recombination (RSS)-like sequences at the wrong chromosomal location, causing a double strand break (DSB) and joining with genuine RSS sequences, either *Ig* or *TCR* genes (Brandt and Roth, 2009). Additionally, activation-induced cytidine deaminase (AID)-related (Muramatsu et al., 1999) translocations may occur via the DSBs of the IgH recombination (Robbiani et al., 2008) as AID is mechanistically associated with the antibody class switch (Muramatsu et al., 2000) as well as antibody diversification (Petersen-Mahrt et al., 2002). Further, enhancement of

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DSBs has been observed through hormone receptor binding to target sites, such as associations with androgen and *TMPRSS2* (a gene with androgen-responsive transcriptional control) facilitating *TMPRSS2-ETS* gene fusions (Kumar-Sinha et al., 2008) equating to site specific, non-random DSBs analogous to those seen for immunoglobulin gene involvement.

Apart from these types of situation, it is still unclear what causes some natural translocations and deletions to occur, particularly nonreciprocal events that are predominant in epithelial tumours (Mitelman et al., 2012), such as by sequence homology between target sites or by nonspecific DSBs repaired by classical nonhomologous end joining (C-NHEJ). Data have been analyzed for a number of settings in which translocations have been analyzed. Further, recent model systems using high throughput sequencing have been analyzed to extract information on interchromosomal events (Chiarle et al., 2011; Klein et al., 2011) and found that DSBs associate with transcribed regions. As our approach to gain further insights into naturally occurring, tumor-associated translocation mechanisms, we have examined the translocation breakpoints (and associated genomic events) of recurrent, nonreciprocal translocations from renal cell carcinoma (RCC), representing each of the three major breakpoint regions on chromosome arm 3p (Kovacs et al., 1987). In RCC, 3p aberrations often result in nonreciprocal translocations, predominantly with chromosome 5, yielding a der(3)t(3;5) chromosome. The examination of the breakpoints shows uniquely intrachromosomal inversion at the breakpoints of each of the RCC translocations. There are no distinct sequences that can be attributed as causative for these translocations. For this to happen, DSBs in *cis* must occur at a time that allows for inversion and ligation before cell division. Presumably, part of the explanation for the loss of one derivative chromosome could be the inversion event. Thus, the most likely explanation for emergence of cancers carrying pathogenic chromosomal abnormalities is clonal selection of afflicted cells from a background of cells with nonspecific repaired DSBs.

MATERIALS AND METHODS

Molecular Copy Number Counting (MCC)

A detailed methodology for MCC has been described (Daser et al., 2006). In brief, genomic DNA (gDNA) is prepared from cells carrying a copy-number variation such as those with nonreciprocal translocation. gDNA is highly diluted and dispensed at less than one genome per aliquot into 96-well plates. In one reaction, a multiplex PCR is carried out using primers representing all the markers in a given panel. The multiplex reaction products are then diluted and split into replica plates and a second round PCR is carried out with seminested primers specific for each marker again in 96-well plates. Seminested PCR products are analyzed by gel-electrophoresis (or by melting curve analysis). The number of wells yielding a PCR product for a given marker is used to statistically calculate the copy-number relative to another marker in the same panel.

Paired-End Sequencing

Illumina mate pair sequencing libraries (Campbell et al., 2008) were constructed using 10 μg genomic DNA with insert size of 3 Kb using standard protocols. 13,386,132 paired end 2x36bp reads were obtained from one lane of an Illumina GAII. The reads were aligned to the human genome (hg19) using eland (Bentley et al., 2008). Putative breakpoints were examined following an approach similar to (Campbell et al., 2008). Briefly, reads which mapped aberrantly (i.e., the two reads of a pair aligned to different chromosomes, or different parts of the same chromosome) were filtered according to read quality, mapping quality, multiple mapping, and mapping to a difficult to map region such as the centromeres. The filtered pairs of reads were then assembled into 72 contigs.

Copy number was calculated using methods as described (Wood et al., 2010). The genome was split into windows corresponding to an average of 400 tumor reads. The number of tumor and a pool of normal blood sample reads aligning to each of these window were counted and tumor normal ratios calculated. Breakpoints were called using the Circular Binary Segmentation algorithm (Venkatraman and Olshen, 2007).

Inverse PCR Cloning

Genomic DNA (1–2 μg) was digested with a suitable restriction enzyme (New England Biolabs) overnight at 37°C in a total volume of 50 μl. The digested DNA was diluted and self-circularized by ligation using T4 DNA ligase (3,200 U; New England BioLabs) in a total volume of 200 μl at 16°C for 16 hr. Circular DNA was precipitated with ethanol and dissolved in TE (40 μl). Totally, 1 μl of the self-circularized DNA mix was used for the primary

PCR reaction using Expand Long Template PCR System (Roche). Where the primary PCR yielded a weak product, 0.5–1 μl of the primary PCR product was used as the template for a second round PCR using an internal forward primer together with the common reverse primer used in the primary PCR in a seminested fashion. Inverse-PCR products were either sequenced directly or cloned, after gel purification using QIAquick Gel Extraction Kit (Qiagen), into pGEM-T Easy vector (Promega).

Sequence Analysis

All sequence annotation and assembly was done using MacVector v10.0. Reference genomic sequences were retrieved from Ensembl (http://www.ensembl.org/index.html). Analysis for DNA repeat sequence was done using RepeatMasker (http://www.repeatmasker.org/).

Genomic PCR

Genomic PCRs were carried out using KOD HiFi DNA polymerase according to the manufacturer's instructions (Novagen). Long-range PCRs were carried out using Extensor Long Range PCR Enzyme Mix (Abgene). PCR for MCC were carried out using AmpliTaq Gold polymerase (Applied Biosystems).

RESULTS

Gene-Free Regions in Nonreciprocal RCC Translocations

We have studied the nonreciprocal t(3;5) translocations in naturally occurring renal cell carcinomas. We used three RCC cell lines (Ebert et al., 1990) that correspond to examples of each of the chromosome 3 breakpoint clusters (Kovacs et al., 1987). Using cytogenetic analysis (unpublished) and SKY-FISH (Strefford et al., 2005) we located these breakpoints to the chromosome 3, short arm (p) regions (SK-RC-9, SK-RC-12, and SK-RC-7) representing breakpoint cluster region (BCR)1, 2 and 3, respectively (illustrated in Supporting Information Fig. S1). The chromosomal translocation breakpoint of SK-RC-7 was examined using the copy number variation (CNV) PCR-based MCC method (Daser et al., 2006) using markers from both chromosome arms 3p and 5q to detect CNV. We found one CNV (that is 1n to 2n) on 5q (location at 106 Mb) (Fig. 1a) (MCC primers used to detect CNV are in Supporting Information Table 1). The relevant genomic sequence was extracted from the human

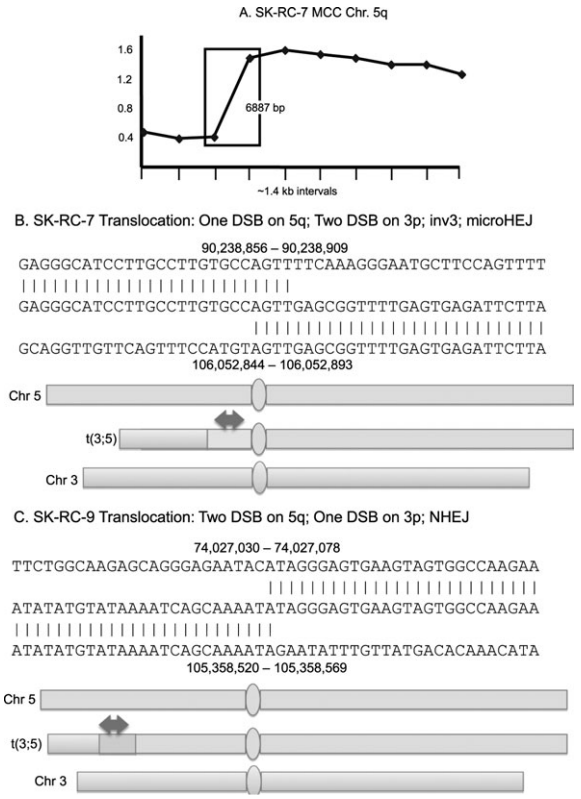

Figure 1. Genomic copy-number variation in regions of chromosomes 3 and 5 in renal carcinoma lines SK-RC-7 and SK-RC-9 CNVs were determined by MCC using SK-RC-7 genomic DNA with primer sets from chromosome 5q (A). The MCC analysis of SK-RC-7 located a CNV between markers 3 and 4 that was used to locate the t(3;5) translocation. The marker spacing and the distance between markers where a CNV occur is on the *x*-axes and the haploid genome equivalents on the *y*-axes. CNV indicated by a box include the base-pairs (bp) separating the relevant MCC markers. The genomic DNA sequence at the translocation breakpoint junction for SK-RC-7 and SK-RC-9 are shown in (B) and (C), respectively. The detected inversion of chromosome 3 or 5 at the t(3;5) are indicated by red double-headed arrow. The proposed mechanism of DNA repair is also indicated (NHEJ, nonhomologous end joining; *micro*HEJ, micro-homology based end joining). The nucleotide positions are based on Ensembl release 64.

genome database and used to design a probe for filter hybridization that located a rearranged 5.5Kb *BamH*I fragment in the SK-RC-7 genome. This fragment was amplified from SK-RC-7 DNA using inverse PCR (Ochman et al., 1988) (the primer sequences are shown in Supporting Information Table 2). The sequence revealed the t(3;5)(p11.1;q21.3) breakpoint junction (Fig. 1b) and the translocation breakpoint genomic fusion was confirmed in SK-RC-7 genomic DNA by conventional PCR amplification across the translocation junction.

Examination of the breakpoint sequence showed several important features. First, no genes are located at or near the chromosome 3 or 5 breakpoints in SK-RC-7 suggesting that transcription per se is not a prerequisite of translocation. In

addition, we observe the presence of a four nucleotide micro-homology between the breakpoint regions of 3p and 5q (Fig. 1b) indicating that the translocation could have occurred, following the DSBs on chromosome 3 and 5, by "micro"-homology-based end joining. The random occurrence of a four base pair sequence would be expected to be every 256 base pairs and thus it is possible, on a random basis, that the so-called micro-homology is coincidental. Even if small homologous recombination events occur, such a mechanism is not mandatory, as suggested by recent high throughput, genome-wide translocation sequencing (Forbes et al., 2011).

Interstitial Inversion at the Junction of Nonreciprocal RCC Translocations

A most significant finding from the sequence data of SK-RC-7 genomic DNA is the unusual occurrence of the sequence at the junction showing an inversion of a region on chromosome arm 3p. This means that this non-reciprocal translocation must have occurred in a cell where at least three unresolved DSBs had occurred to yield the structure shown in Figure 1b. Given this finding of a complex chromosomal fragmentation and reorganization in SK-RC-7 (representing BCR3), we re-evaluated the previously noted SK-RC-9 translocation junction sequence, representing BCR1 (Daser et al., 2006). These sequence data also show that the mechanism of SK-RC-9 translocation is from DSBs and classical NHEJ, causing the translocation junction of chromosome 5 to be inverted (Fig. 1c).

To further examine the frequency of intrachromosomal inversion with nonreciprocal translocations, we defined the t(3;5) translocation breakpoints of a RCC carrying a translocation of the third breakpoint cluster region on chromosome 3 (SK-RC-12 representing BCR2). A first round of MCC was carried out to scan a region of chromosome 3, bands 3p13–3p11.1, for CNV at low resolution (Fig. 2a) (the primer sequences used are listed in Supporting Information Table 3). This located a CNV 1n to 2n between markers 17 and 18 that potentially represented the t(3;5) breakpoint region. A further two rounds of MCC (Figs. 2b and 2c), using PCR primers for markers at progressively increasing resolution, located two separate CNVs on the short arm of chromosome 3.

Genomic analysis showed restriction fragment variation in the SK-RC-12 DNA compared with control DNA and inverse PCR, using self-ligated *Kpn*I and *Pst*I digested genomic DNA, yielded fragments that sequence analysis showed corresponded respectively to 6.2 Mb and 0.29 Mb deletions rather than to the t(3;5) translocation breakpoint (Figs. 2d and 2e). Examination of the sequences at the deletions shows that, analogous to the translocation breakpoints, one of these seems to have occurred by DSB and C-NHEJ and one by DSB followed by micro-HEJ via a region of 6 base pairs homology (Fig. 2e). The 6.2 Mb deletion resulted in loss of *DUTT1*/*ROBO1* and *ROBO2* genes but genomic PCR analysis showed hemizygous deletion of *DUTT1*/*ROBO1* gene in SK-RC-12 (also observed in SK-RC-7, Data not shown). In addition, the 3′ deletion junction of the 6.2 Mb deletion falls within the *GBE1* gene but no genes are involved in the 5′ deletion break. However, the 0.29 Mb deletion also affects the gene *GBE1*, as the 5′ deletion breakpoint falls within the intronic region located between exons 2 and 3. These deletions result from classical deletions followed by rejoining without inversion and they do not result in gene fusion.

We employed an alternative method to locate and clone the t(3;5) breakpoint in SK-RC-12 based on Next Generation DNA sequencing to produce CNV karyogrammes (Wood et al., 2010). Using paired end sequencing (Campbell et al., 2008) we were able to delineate the t(3;5) breakpoint by comparing CNV karyogrammes to a reference human genome. Three RCC cell lines were analyzed, two with t(3:5) translocations (SK-RC-12 and SK-RC-48) and one with karyotypically normal chromosome 3 as a control (SK-RC-45). CNV were apparent in SK-RC-12 and SK-RC-48 for chromosome 3 short arm (Figs. 3a and 3b) whereas only small loss of material seems to have occurred on SK-RC-45 chromosome 3p (Fig. 3c), in a region associated with all the 3p deletions that we observed in RCC. Conversely, SK-RC-12 and SK-RC-45 have a 1n:2n shift on chromosome 5 while SK-RC-48 has a duplication of chromosome arm 5p. By comparing to the reference genome, we identified four independent overlapping paired end reads (Supporting Information Fig. S2) spanning the t(3;5) breakpoint region; these sequence data allowed genomic PCR of SK-RC-12 DNA to amplify a fragment spanning the translocation (shown in Fig. 3d). The fragment sequence reveals a t(3;5) translocation junction in which an interstitial inversion of chromosome arm 3p has occurred by DSB and C-NHEJ following two DSB on chromosome arm 3p and one on chromosome arm 5q. The t(3;5) (p13;q21.1) breakpoint

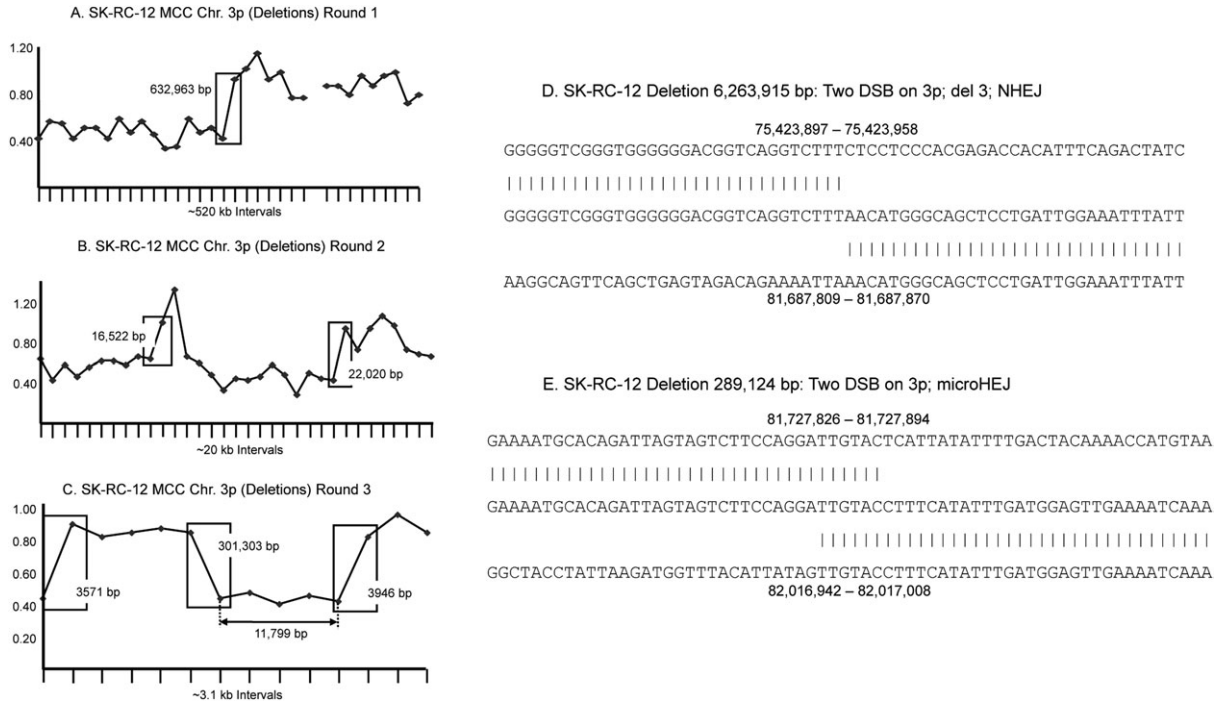

Figure 2. Genomic DNA sequences at the breakpoint junctions of interstitial deletions in a renal cell carcinoma line SK-RC-12. MCC was used to identify genomic rearrangements in the DNA of SK-RC-12 at progressively shorter spacing; the rearrangements were verified by Southern filter hybridisation, and inverse PCR cloning was used to obtain sequencing templates to identify the changes at the nucleotide level. A–C: MCC copy number variation plots using SK-RC-12 DNA with PCR primer pairs set at 520Kb (A), 20kb (B), or 3.1Kb (C). D and E: Sequences of deletion breakpoints in SK-RC-12 (middle sequences) are aligned with the 5′ and 3′ deletion junctions from the chromosome 3 reference sequence. The proposed mechanism of DNA repair is also indicated (NHEJ, nonhomologous end joining; *micro*HEJ, micro-homology based end joining). The nucleotide positions are based on Ensembl release 64.

falls towards the end of the *SHQ1* gene on chromosome 3 (just before exon 11) but the break on chromosome 5 does not involve a gene.

DISCUSSION

Our findings with the unbalanced translocations of RCC show that the regions where the breakpoints occur do not always correspond directly to regions of gene expression and do not result in gene fusion. In recent studies, genome-wide translocations were preferentially associated with transcribed regions, particularly start sites (Klein et al., 2011) supporting an accessibility model that links chromatin "open" structures to propensity to undergo translocation. Naturally occurring translocations that occur because DSBs are caused by noncell autonomous factors (e.g., carcinogens, radiation), rather that RAG/AID-mediated changes, can presumably occur independently of transcription. Other physiological situations, such as described in prostate cancer where aberrant chromosomes may occur from DSBs being induced by androgen receptor binding (Williamson and Lees-Miller, 2011) recruiting AID and a LINE-1 endonuclease, locate specific chromosomal abnormalities such as produce *TMPRSS2-ERG* fusion genes (Lin et al., 2009). In the cases we describe here in the renal cancers at presentation of disease, there must be at least three unresolved DSBs that occur simultaneously or sequentially to form the aberrant chromosomes for which the causation is unknown. Transcribed regions can be involved at translocation junctions, but this is not mandatory questioning the concept that transcription and translocation-prone DSBs are linked.

Chromosomal translocation between nonhomologous chromosomes have been known for many decades and their association with cancer outcome is established (Mitelman et al., 2007). DSBs and NHEJ as precepts of chromosomal rearrangements is an established concept (Richardson and Jasin, 2000; Weinstock et al., 2006). The belief that intranuclear, spatial organisation of chromosomes limits interchromosomal translocations is questioned by the panoply of translocations observed between *MLL* on chromosome 11, band q23 and almost every chromosome arm in human leukaemias (Daser and Rabbitts, 2005; Liu et al., 2009) or between EWSR1 and varying fusion partners in sarcomas

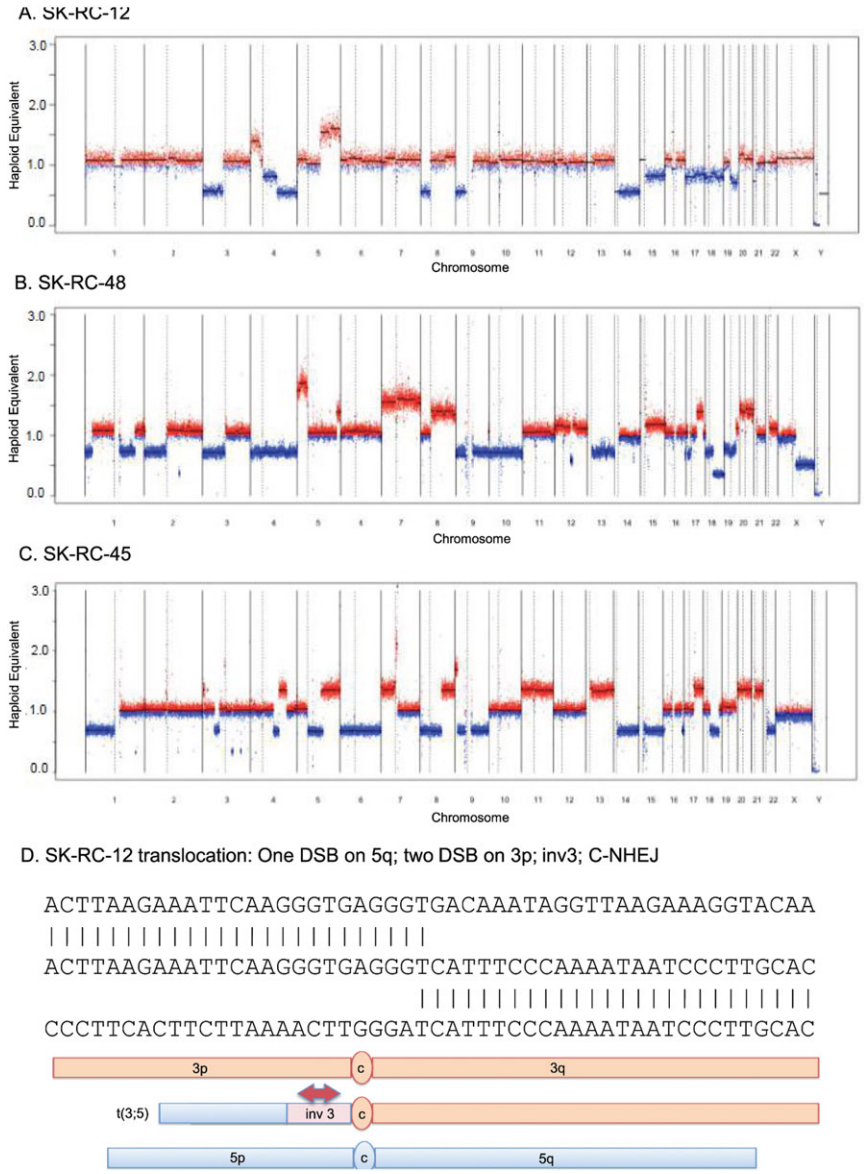

Figure 3. Whole genome copy-number variation karyogramme and t(3;5) breakpoint sequence of renal carcinoma cell line SK-RC-12 DNA SK-RC genomic DNAs were subjected to copy number analysis following NGS deep sequencing paired end sequence reads. Gross copy-number variations per haploid genome (*y* axis) are shown for each chromosome at 150 Kb intervals (*x* axis); Overlay of haploid genome equivalents for points along each chromosome (red dots = tumour:normal ratio >1; blue dots = tumour:normal ratio <1). (respectively, panel A: SK-RC-12; panel B: SK-RC-48, and panel C: SK-RC-45). D: From the CNV karyogramme, the t(3;5) nonreciprocal chromosomal translocation is evident in SK-RC-12. Deep sequencing was used to identify genomic rearrangement, verified by Southern filter hybridization, and inverse PCR cloning used to obtain sequencing templates. The t(3;5) breakpoint sequence is shown, aligned to sequences on chromosomes 3 or 5 and the nucleotide sequence locations shown. The detected inversion of chromosome 3 at the t(3;5) is indicated by red double-headed arrow.

(Taylor et al., 2011). Nonetheless, mouse data from situations using ionizing radiation to induce DSBs showed induction of chromosomal aberrations that predominate in regions of spatial proximity (Zhang et al., 2012). *Cis*-acting rearrangements were commonly found and analogous mechanisms could account for the multiple DSB required in these renal cell carcinomas to cause local intrachromosomal inversion events. It is also therefore probable that inversion and chromosome loss mechanisms are linked at least in those cases where inversion occurs. This is presumably a reflection of the mechanism of the nonreciprocal translocation characteristic of renal cell carcinoma in which the der(3;5) is lost. If the segments of chromosome 3 and 5 join without inversion, the consequence is dicentric and acentric chromosomes. The latter would be lost at cell division while the dicentric chromosome could

survive and subsequently lose one centromere in the inversion event.

Amalgamated, these sequence organizational findings show that a variety of genomic mechanisms can aberrantly result in translocations, inversions and deletions but that sequence specificity or homology are marginal contributors. Our data show that the RCC nonreciprocal translocations, and interstitial deletions, occur by DSB and C-NHEJ (and sometimes with microhomologies retained following the resolution of the DSBs). If transcription is not a regional factor in the DSBs, the involvement of repeat genomic sequences also seems an unlikely common cause. Repeats are observed near the breakpoints of the deletions and translocations in these renal cancers (illustrated in Supporting Information Fig. S3), but, in only one case, was this at the actual breakpoint (SK-RC-12, Supporting Information Fig. S3D).

In the majority of tumors with translocations, these can be attributed to natural processes that normally govern chromosome stability (i.e., intrachromosomal repair of DSBs) working incorrectly to join unrepaired DSBs and cause translocations. One likely mechanism is a model where breaks occur first (Meaburn et al., 2007) but where the spatial setting of chromosomes is relevant (Zhang et al., 2012). Once the DSBs have coincidently occurred on distinct locations, cancer arises by clonal selection because the end joining (normally controlled to rejoin the correct ends) causes a carcinogenic, interlocation join. Clonal selection is ultimately the powerful biological process behind tumor outgrowth following chromosomal translocation or other chromosome abnormality with similar outcome, whether this occurs as a consequence of essentially random events (e.g., ionizing radiation) or by "directed ones" such as DSBs mediated by RAG recombinase, AID or by transcription factors like androgen receptors. In this model, while the frequency of chromosomal translocations or inversions may not necessarily be uncommon as shown for chromosome 14 breakpoints in normal individuals (Welch et al., 1975), the cancer-causing ones must be rare to account for the infrequency of cancer in the cellular population. It should be borne in mind however that t(14;18) translocations, the hallmark of follicular lymphoma, are commonly seen in tonsillectomy biopsies (Limpens et al., 1991).

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