Human Papillomavirus Type 16 Integrations in Cervical Tumors Frequently Occur in Common Fragile Sites

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Abstract

The development of cervical cancer is highly associated with human papillomavirus (HPV) infection. HPV integration into the genome of infected cervical cells is temporally associated with the acquisition of the malignant phenotype. A relationship between the sites of HPV integration in cervical cancer and the position of the common fragile sites (CFSs) has been observed at the cytogenetic level. To explore this relationship at the molecular level, we used a PCR-based method to rapidly isolate cellular sequences flanking the sites of HPV16 integrations in primary cervical tumors. Human bacterial artificial chromosome clones were isolated based on these flanking sequences and used as probes for fluorescence in situ hybridization on metaphases derived from cells cultured in the presence of aphidicolin. Our data demonstrate that HPV16 integrations in cervical tumors frequently occur within CFSs at the molecular level. In addition, we have determined the precise molecular locations of the CFSs FRA6C and FRA17B.

Introduction

Fragile sites are specific chromosomal loci that, under appropriate culture conditions or exposure to certain chemical agents, nonrandomly exhibit the tendency to form chromosome and/or chromatid gaps and breaks and other chromosomal abnormalities. There are 89 recognized CFSs distributed throughout the human genome. The vast majority of these sites are induced in response to aphidicolin, a DNA polymerase inhibitor. To date, 4 aphidicolin-sensitive CFSs have been cloned: FRA3B, FRA7G, FRA7H, and FRA16D (1–5). The cloning and partial sequencing of these CFSs have not yet revealed the mechanistic role in the development of chromosomal abnormalities that CFSs are preferential targets for sister chromatid exchange (6), translocations and deletions (7), intrachromosomal gene amplification (8), and the integration of plasmid DNA and tumor-associated viruses (1, 3, 9). The genomic instability that occurs at CFSs has led to the hypothesis that CFSs and the genes that reside within them play a role in the development of chromosomal abnormalities and mutations in human cancer (10).

Cervical cancer is the second most common cause of cancer-related mortality in women worldwide. Most cervical cancers are squamous cell carcinomas that develop through a distinct morphological progression. Progression from a premalignant lesion to invasive cancer usually takes years to occur, indicating the multistep process of cervical cancer development (11). Cervical cancer is highly associated with high-risk HPV infection, and HPV sequences are detected in nearly every tumor examined. HPV16 and HPV18 are the prototypical high-risk HPV types and are found in the majority of cervical tumors. Several lines of evidence indicate that high-risk HPV integration is an important event in the progression of cervical cancer. In premalignant cervical lesions, the HPV genome is typically maintained in its episomal form. However, the majority of invasive cervical carcinomas contain HPV DNA that has integrated into the host cell genome. Thus, the integration event is temporally associated with the acquisition of the malignant phenotype (11). During the process of integration, deletions typically occur within the HPV genome. However, the oncogenic E6 and E7 open reading frames are virtually always retained, underscoring the importance of the continued expression of these genes in the maintenance of the malignancy. The integration event may also contribute to the progression of cervical cancer by disrupting the coding sequence, splicing, or expression of genes at or near the site of integration. Thus, specific integrations may be selected for by the tumor, leading to the single clonal integration observed in most tumors (11).

We propose that CFSs are preferential targets for HPV integration in cervical tumors. Until recently, it was thought that the sites of HPV integration were distributed randomly throughout the genome. However, cytogenetic mapping of multiple integration sites suggested that they occurred preferentially in bands containing CFSs (12). More recently, molecular evidence of this relationship arose when the sequence of the FRA3B region at 3p14.2 revealed an HPV integration event that occurred preferentially in bands containing CFSs (13). Several lines of evidence indicate that high-risk HPV integration is an important event in the progression of cervical cancer. In premalignant cervical lesions, the HPV genome is typically maintained in its episomal form. However, the majority of invasive cervical carcinomas contain HPV DNA that has integrated into the host cell genome. Thus, the integration event is temporally associated with the acquisition of the malignant phenotype (11). During the process of integration, deletions typically occur within the HPV genome. However, the oncogenic E6 and E7 open reading frames are virtually always retained, underscoring the importance of the continued expression of these genes in the maintenance of the malignancy. The integration event may also contribute to the progression of cervical cancer by disrupting the coding sequence, splicing, or expression of genes at or near the site of integration. Thus, specific integrations may be selected for by the tumor, leading to the single clonal integration observed in most tumors (11).

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Materials and Methods

Samples. HPV16-positive cervical tumor specimens were obtained from seven patients. DNA extraction and HPV typing were performed as described previously (13). All tumors were grade 2 to grade 4 squamous cell carcinomas. The HPV16 cervical cancer cell line SiHa was obtained from the American Type Culture Collection. Cells were grown as recommended, and DNA extraction was performed using standard protocols.

PCR Conditions. RS-PCR (14) is a direct method that rapidly retrieves unknown sequences adjoined a known sequence. RS-PCR uses primers (RSO) that recognize a given restriction enzyme recognition site, anneal to that site,
and promote DNA synthesis in a PCR. The RSOs consisted of (5' to 3') a T7 phage promoter sequence, a completely redundant sequence of 10-base length, and the sequence of a restriction enzyme recognition site. Four RSOs were used that were specific for the restriction enzymes BamHI, EcoRI, Sau3AI, and TaqI. To retrieve an unknown sequence, PCR was performed with a primer specific for the known HPV16 sequence (Table 1) and individual RSOs. Subsequently, a nested PCR was carried out using an aliquot of the first PCR as template, an internal primer from the known HPV16 sequence (Table 1), and the same RSO. Two pmol of each specific primer and 20 pmol of the RSO primer were combined with 100 ng of SiHa or cervical tumor DNA, 1× PCR buffer (Boehringer Mannheim) containing 1.5 mM MgCl₂, 200 μM deoxynucleotide triphosphates, and 0.5 unit of Taq polymerase (Boehringer Mannheim) in a 20-μl reaction volume. Three replicates of 1 min at 94°C, 2 min at 45°C (first round of PCR) or 55°C (nested PCR), and 3 min at 72°C, followed by 10 min at 72°C were performed. One μl of the product from the first round of PCR was used as template for the nested PCR. The PCR products obtained using this method were directly sequenced as described below. Conditions for PCR shown in Fig. 2 were performed with 50 ng of template, 10 pmol of each primer, 1× PCR buffer (Boehringer Mannheim) containing 1.5 mM MgCl₂, 200 μM deoxynucleotide triphosphates, and 0.5 unit of Taq polymerase (Boehringer Mannheim) in a 25-μl reaction. The cycling conditions were as follows: 30 s at 94°C, 30 s at 55°C, 30 s at 72°C for 30 cycles, followed by a 10-min extension at 72°C.

**Sequencing.** One hundred ng of PCR products were treated with 1 unit of exonuclease I (United States Biochemical) for 15 min at 37°C, followed by 15 min at 80°C, and then treatment under the same conditions with 1 unit of shrimp alkaline phosphatase (Roche). The template was combined with 3.2 pmol of the appropriate sequencing primer (Table 1). Sequencing was performed by the Mayo Clinic Molecular Core Facility.

**Radiation Hybrid Screening.** Primers were designed to genomic sequences flanking the sites of HPV16 integration (Fig. 2). These primers were used to screen the Stanford G3 Radiation Hybrid Mapping Panels (Research Genetics) as described by the manufacturer. Chromosomal locations based on linked markers and LOD scores were obtained from the Stanford University web site.

**BAC Screening and Isolation.** The same primers used for the radiation hybrid screening were used to screen the CITB Human BAC DNA Pools–Release IV (Research Genetics) as recommended. Positive BAC clones were obtained from Research Genetics. The BAC DNA isolation procedure was adapted from the Qiagen Maxi-prep protocol. The precise protocol is available upon request.

**Computer Searches.** BLASTN searches with the cellular sequences flanking the sites of HPV16 integration were performed on the National Center for Biotechnology Information website.

**FISH.** Phytohemagglutinin antigen-stimulated peripheral blood lymphocytes were cultured for 72 h, arrested with Colcemid, and fixed according to standard procedures (15). Aphidicolin (0.4 μM; Sigma) was added to the culture for the final 24 h to induce CFS expression. Metaphase preparations were dropped onto glass slides. BAC DNA (1 μg) was labeled with biotin-16-DUTP by nick translation (Boehringer Mannheim) as recommended by the manufacturer. Labeled BAC probes were hybridized to the metaphase spreads using standard procedures (15). Hybridized probes were detected with a fluorescein detection kit and counterstained with propidium iodide or 4’,6’-diamidino-2-phenylindole (Oncor). Hybridization signals were detected with a Zeiss Axioskop microscope, and images were captured using the IP LabSpectrum P software (Signal Analytics Corp.).

**Results.**

**Isolation of Sequences Flanking the Sites of HPV16 Integration.** RS-PCR is a PCR-based technique for amplification of unknown nucleotide sequences next to known nucleotide sequences (14). RS-PCR uses specialized PCR primers that contain restriction site recognition sequences in conjunction with primers that are specific to the known nucleotide sequences (see “Materials and Methods”). To determine whether RS-PCR was applicable to rescuing sequences adjacent to the sites of HPV16 integration in cervical tumors, we first tested the technique on DNA isolated from the HPV16-positive cervical tumor cell line SiHa. SiHa contains a single HPV16 integration on chromosome 13, and the sequence flanking this integration has been determined previously (16). We performed RS-PCR on SiHa genomic DNA, and a single product was observed that corresponded to the expected size. Subsequent sequencing of this product confirmed that the sequence was identical to that published previously (data not shown).

We next used RS-PCR to screen genomic DNA samples obtained from seven HPV16-positive cervical tumors: CC171, CC192, CC194, CC212, CC224, CC226, and CC236. The HPV16-specific primers used for the screening are listed in Table 1. Primers were designed to take advantage of the fact that upon HPV integration, the HPV E6 and E7 open reading frames are almost always retained, whereas other portions of the HPV genome may be deleted. Eight sets of three primers (one for primary RS-PCR, one for nested RS-PCR, and one for sequencing) were designed at ~750-bp intervals extending out in both directions from the E6 and E7 open reading frames. Four RSO primers with sequences complementary to BamHI, EcoRI, Sau3AI, and TaqI recognition sites were used in combination with the HPV16-specific primers. In total, 32 primary RS-PCR reactions were performed (4 RSO primers × 8 primary HPV16-specific primers), followed by 32 nested RS-PCR reactions (same 4 RSO primers × 8 nested HPV16-specific primers) for each tumor. Seven μl of each nested RS-PCR reaction were run on 2% agarose gels. Typical nested RS-PCR results are shown in Fig. 1.

Using RS-PCR and subsequent sequencing of the products, cellular sequences flanking HPV16 sequences were obtained for all seven tumors. Three tumors (CC194, CC212, and CC224) had an HPV integration within or adjacent to an Alu repetitive element. One tumor (CC236) had an HPV integration into a sequence with high homology to the α satellite family of tandemly repeated DNAs typically found in centromeric regions of chromosomes. We were unable to assign the chromosomal location of the HPV16 integrations in these tumors because site-specific primers could not be constructed because of the repetitive nature of the flanking sequences. Three tumors (CC192,
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CC171, and CC226) had HPV16 integrations into specific, nonrepetitive regions of the genome. The hybrid HPV-cellular sequence obtained for these three tumors and the sites of primers designed to the flanking sequence are shown in Fig. 2a. The primers designed within the cellular flanking sequences were used to amplify a specific product from normal human DNA. Products of the expected size and sequence were obtained in all three cases (data not shown). To determine the specificity of the junctional sequence to the tumor of interest and to verify that the products were not attributable to a RS-PCR artifact, amplification between the HPV16-specific primers used in the primary RS-PCR and the upstream flanking sequence-specific primers was performed using the appropriate tumor DNA as template. A single specific band of expected size was generated for all three tumors (Fig. 2b). Subsequent sequencing of the PCR products generated the expected HPV16-normal cellular junctional sequence, indicating that the integration was specific to the tumor of interest (data not shown).

Identification of the Chromosomal Location of HPV16 Integration for CC171, CC192, and CC226. The normal cellular sequences flanking the three HPV16 integration events were used to search the nr and dbest databases with BLASTN. No significant matches were observed for the flanking sequences obtained from CC171, indicating that this integration occurred in a region of the genome that has not been sequenced.

The flanking sequence for CC192 did not have any significant homology to sequences in the nr database. However, significant hits were obtained within the dbest database. Human expressed sequence tag sequence T77269 had the highest homology to the flanking sequence. This expressed sequence tag represents an IMAGE clone that belongs to the Unigene entry Hs.8015, which corresponds to a ubiquitin-specific protease on chromosome 1q21. The mRNA sequence of this gene was published recently and named USP23 (17). However, based on our data, this published sequence is not complete. Our data, which has been confirmed by IMAGE clone sequencing, reverse transcription-PCR, and Northern blot analysis, indicate that 222 bp (74 amino acids) should be inserted at bp 1473 of the published sequence. The HPV16 integration in this tumor occurred within this additional coding sequence. Alternative splicing may be the explanation for this difference, because reverse transcription-PCR using primers flanking the additional sequence produced two bands of distinct sizes. In addition, Northern blots using probes including this additional sequence revealed two differentially spliced products (data not shown).

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The flanking sequence for the HPV16 integration in CC226 matched perfectly to bases 117874–118046 of BAC hRPK.60_A.24 (GenBank accession number NT 000830), which has been mapped to chromosomal band 17q23.

To determine the chromosomal location of the HPV16 integration in CC171, primers CC171FlankD/CC171FlankU (Fig. 2b) were used to screen the Stanford G3 radiation hybrid mapping panels. The results demonstrated that the markers with the highest LOD scores (SHGC-20333, SHGC-34186, and SHGC-1841 with LOD scores of 12.53, 11.77, and 9.15, respectively) mapped to chromosomal band 6p22.2. In addition, CC171FlankD/CC171FlankU and CC192FlankD/CC192FlankU were used to screen the Research Genetics CITB human BAC pools to identify single BAC clones that represented the preintegration sequences. Single BAC clones, CITB.564_C.7 and CITB.31_K.11, were identified for CC171 and CC192, respectively. These BAC clones, in addition to hRPK.60_A.24 were obtained and cultured, and DNA was extracted. The BAC DNA from these clones was labeled and used as a probe for FISH on slides with metaphase spreads obtained from a normal individual. BAC clones CITB.564_C.7, CITB.31_K.11, and hRPK.60_A.24 hybridized to chromosomal bands 6p22.2, 1q21, and 17q23, respectively, confirming the radiation hybrid and database mapping results (data not shown).

**Determination of the Integration Site Relative to CFSs in CC171, CC192, and CC226.** The locations of all three HPV16 integrations determined above also correspond to chromosomal bands known to harbor CFSs (Fig. 3a; Ref. 10). To determine whether the HPV16 integrations occurred within these CFSs at the molecular level, the BAC clones representing the preintegration sequence for each of the three tumors were used as FISH probes. Metaphase spreads were prepared from peripheral blood lymphocytes treated with 0.4 μM aphidicolin to induce the expression of CFSs. After hybridization with each BAC clone individually, metaphases were examined for CFS expression. Because an individual BAC clone is typically smaller than the region of breakage within a CFS, the BAC may hybridize proximal to, distal to, or cross the site of aphidicolin-induced breakage, depending on where within the CFS the breakage occurs. To determine whether an individual clone is contained within a CFS, signals must be seen that are both proximal and distal to the site of breakage in different metaphases. For this reason, it is necessary to examine the distribution of hybridization signals across many metaphases that show expression of the CFS of interest. At least 20 breaks at each CFS were counted for each BAC hybridization. The data demonstrate that the HPV16 integrations in tumors CC171 and CC226 occurred within FRA6C at 6p22.2 and FRA17B at 17q23.1, respectively, whereas the integration in tumor CC192 was near the FRA1F on 1q21 but not within it (Fig. 3a). Representative FISH images demonstrating BAC hybridizations, both proximal and distal to aphidicolin-induced breakage, at the CFSs FRA6C and FRA17B are shown (Fig. 3b).

**Discussion**

The 89 CFSs are distributed throughout the human genome and may comprise as much as 1% of the total DNA in the genome. Their precise role in normal cells and the role that they play in the development of cancer are unknown, but there has been increasing evidence that these sequences may be involved in a number of important processes that occur during cancer development. These include translocations and deletions (7), intrachromosomal gene amplification (8), and the integration of plasmid DNA (9) and tumor-associated viruses (1).

The importance of the site of high-risk HPV integration in cervical tumors can be determined by examining the randomness of the integration events. If HPV integration is truly random, it is unlikely that the integration event contributes to the malignant phenotype (other than overexpression of the E6 and E7 oncoproteins). If HPV integration is nonrandom, there are a number of important questions to ask about this relationship including: (a) Are there genes that are specifically disrupted or deregulated by the integration event that are important contributors to the development of cervical tumors? (b) Are CFSs preferential targets of HPV integration? and (c) If so, are there genes within the CFSs that are important targets that contribute to the malignant phenotype? In an attempt to answer these questions, and to gain insights into the earliest processes that occur in the development of cervical tumors, we have studied the relationship between the sites of HPV16 integrations in cervical tumors and CFSs.

In this report, we describe our results using RS-PCR, a rapid method for isolating sequences flanking the sites of HPV16 integrations (14). The utility of this technique was demonstrated on the well-characterized integration observed in the cervical cancer cell line, SiHa. Fusion sequences were then detected in all primary tumors examined. However, an unexpectedly high number of integrations occurred within or near repetitive elements, particularly Alu elements. This reduced the number of tumors that were informative as to their HPV16 integration site. It should be noted that the HPV16 integration observed within the CFS FRA3B also occurred adjacent to an Alu sequence (1). There is no significant homology between the HPV16 genome and consensus Alu sequences, ruling out the possibility that homologous recombination is involved as an insertional mechanism. Three of the integrations occurred within unique sequences, which enabled us to identify unique BAC clones that spanned these three integration sites. We localized the sites of integration using both radiation hybrids and with FISH, using the BAC clones as hybridization probes. All three integrations occurred within chromosomal bands known to contain CFSs, and the FISH-based analysis revealed that two of the three integrations occurred within a CFS at the molecular level. Therefore, this work has enabled us to precisely pinpoint two previously uncharacterized CFSs, FRA6C and FRA17B. We are currently in the process of identifying HPV16 integrations sites from a large number of primary cervical tumors. This work may enable us to precisely pinpoint a number of the other CFSs. An analysis of a much larger series of cervical tumors should enable us to determine the percentage of integrations that actually do occur within CFSs and also whether there will be any single CFS that receives a greater proportion of integrations than expected by chance.

However, the potential specificity of HPV16 integrations within the CFSs is only part of the story. HPV integration events in previously analyzed tumors have clearly demonstrated that cellular genes at or near the integration site could be disrupted or deregulated by a variety of mechanisms. Integrations within genes, deletions, and amplifications of sequences adjacent to the integration, as well as hybrid transcripts formed between HPV sequences and sequences at the site of integration, have all been shown to occur at the integration sites in cervical tumors (1, 18–22). Whether these genes are specific mutational targets in these tumors or merely bystanders that do not contribute to the malignant phenotype remains to be seen. Our current analysis of the sequences flanking the sites of HPV16 integration indicates that there are genes residing at the site of integration in at least two of the three tumors whose integrations we could precisely map. The observation that the integration in CC192 occurred within the coding sequence of the USP23 gene suggests that this gene may be a target of mutation in cervical and/or other tumor types. Analysis of the DNA sequence of hRPK.60_A.24 (which spanned the integration site from tumor CC226) indicates that a cellular gene with homology to RNA-binding proteins partially resides within this BAC, and that
the integration event in this tumor took place within an intron of this gene. We are currently analyzing these genes for altered expression in cervical tumor cell lines and primary tumors to determine whether they are specifically mutated or deregulated. The integration in the third tumor, CC171, occurred within chromosomal band 6p22.2, a region of high LOH in cervical tumors (23). This may indicate that there is another important gene at or near this integration site that plays a role in the initiation or progression of cervical cancer.

The finding that HPV16 integrations frequently target the CFSs in cervical tumors provides further evidence in support of the biological relevance of CFSs. It seems highly likely that other tumor-associated DNA viruses may also preferentially target CFSs as sites of integration. We are currently exploring this in other systems, including hepatitis B integrations in hepatocellular carcinomas and papillomavirus integrations in skin cancers. It still remains to be determined whether the CFSs are the targets of viral integration merely because of their innate instability or if integrations into the CFSs specifically contribute in a mutational or mechanistic way to the evolution of these tumors.

References


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