Human papilloma virus (HPV) integration signature in Cervical Lesions: identification of MACROD2 gene as HPV hot spot integration site.

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Abstract

Background

High-risk HPV is clearly associated with cervical cancer. Integration of HPV DNA into the host genome is considered a key event in driving cervical carcinogenesis. However, the mechanism on how HR-HPV integration influences the host genome structure has remained enigmatic.

Methods

In our study, 25 DNA samples including 11 from fresh-frozen cervical carcinomas and 14 from fresh-frozen high-grade squamous intraepithelial lesion (HSILs) were detected using the method of HPV capture combined with next generation sequencing.

Results

We calculated the frequency in each viral gene or region and found that breakpoints were prone to occur in L1 and L2 instead of E2 in the cervical cancer ($P = 0.0004$ and $P = 5.15\times10^{-40}$) and HSIL group ($P = 2.1\times10^{-32}$ and $P = 7.06\times10^{-13}$). The results revealed that HPV16 showed a strong tendency towards intronic region (P = 5.02×10−64) but a subtle tendency towards intergenic region (P = 0.04). The most frequent integration site was in the MACROD2 gene (introns 2, 4, 5, 6, 8 and 9), which in MACROD2 functional domain.

Conclusion

Our results revealed that MACROD2 is HPV hot spot integration site in cervical lesions, and its deficiency alter DNA repair and sensitivity to DNA damage thought impaired PARP1 activity resulting in chromosome instability.

Introduction

Cervical cancer is the fourth most frequently diagnosed cancer and the fourth leading cause of cancer death in women, with an estimated 604,127 cases and 341,831 deaths in 2020 worldwide[1]. High risk human papillomavirus (HR-HPV) persistent infection is the main causative factor of cervical cancer and intraepithelial neoplasia (CIN), HR-HPVs are the causative agents of cervical cancer and have been detected in 99.7% of cervical cancers[2]. The infection with HPV 16 and 18 high-risk types account for more than 80% of cervical cancer incidence[3]. Integration of HPV DNA into the host genome is considered a key event in driving cervical carcinogenesis [4]. The increase of both integration rate and number from CINs to cancer highlights their potential values as predictors of disease progression[5]. The integration occurs in regions of micro-homology among the HPVs and host genome. Viral genome integration events usually result in dysregulation of E6 and E7 gene expression compared to that expressed from extrachromosomal viral genomes[6]. As mentioned above, most integration events result in expression of a spliced viral-cellular transcript[7]. These fusion transcripts are very often more stable than their viral counterparts, yet again increasing HPV oncogene expression[8]. However, the mechanism on how HR-HPV integration influences the host genome structure has remained enigmatic.

HPV integrations may occur either within UTRs (5’ UTR: 12.6%, or 3’ UTR: 15.3%), within genic regions (exons: 2.7%, introns: 39.6%) or within intergenic regions (29.7%), indicating that HPV-based disruptions may lead to the introduction of aberrant gene promoters, aberrant enhancers and/or aberrant microRNA expression patterns, thereby inactivating tumor-suppressor genes, activating proto-oncogenes, inactivating DNA repair genes and promoting genomic instability[9]. Protein

expression from *FHIT* and *LRP1B* was downregulated when HPV integrated in their introns[10]. Previous study revealed that the patterns of HPV integration in RNA and DNA samples different significantly. *FHIT*, *KLF5*, and *LINC00392* were the hotspot genes integrated by HPV in the DNA samples. *RAD51B*, *CASC8*, *CASC21*, *ERBB2*, *TP63, TEX41*, *RAP2B*, and *MYC* were the hotspot genes integrated by HPV in RNA samples[11]. *SLC26A2* protein expression was aberrantly high, supposedly as a result of its *HPV16 E1- SLC26A2* fusion and increased transcription[12]. *Jinmiao Chen* et al. reported human-HPV chimeric RNAs *GFRAL-HPV16 E5* and *OS9-HPV16 E2*[13].

Despite increased attention on HPV integration hotspots, the characteristic of HPV integration and the relationship between HPV integration and cervical cancer remained elusive. In this study, we focus on integration sites analysis of 25 HPV16- positive cervical lesion samples. Our data revealed a hotspot of HPV integration at *MACROD2*, involved in impaired *PARP1* activity and chromosome instability. Transcription of *HPV16-MACROD2* gene fusions from the site of genome integrations was showed through transcriptome sequencing. Our study could further help to gain insights into the characteristic of HPV integration in DNA and RNA samples and provide theoretical basis for understanding the mechanism of tumorigenesis.

Material And Methods

Study population and specimen collection

A total of 11 fresh tissue specimens were collected from patients with cervical cancers who had undergone surgeries, and 14 cervical biopsy specimens were collected and diagnosed with high-grade squamous intraepithelial lesion (HSIL) from Yantai Yuhuangding Hospital, Shandong province, China, 2021.

Individual informed consent had been collected from all study participants. This study received ethical approval from the Institutional Review Board of our hospital. All experiments were performed in accordance with relevant guidelines and regulations.

Genomic DNA isolation, HPV typing

DNA from the cervical cast-off cells were extracted by a TIANamp Genomic DNA Kit (No: 3304-9) according to the manufacturer’s procedure. Human papillomavirus genotyping was conducted using an HPV GenoArray test kit (HybriBio Ltd).

HPV integration detection

HPV probes were designed according to the full-length genome of 32 HPV types by MyGenostics (MyGenostics, Baltimore, MD, USA). 18 HPV types (16,18,26,31,33,35, 39,45,51,52,53,56,58,59,66,68,73,82) were analyzed in subsequent HPV assays. The overall experiment was conducted according to the manufacturer's protocol.

Detecting integration breakpoints by RNA-seq

We selected 3 cervical cancers with hot-spot genes detected by HPV capture technology combined with next generation sequencing and sufficiently high-quality RNA. RNA-seq libraries were sequenced as paired-end 90-bp sequence tags using the standard Solexa pipeline. We carried out the analysis of integration sites using the transcriptome data according to a previous method[10].

Statistical analysis

Fisher's exact test was chosen for statistical analysis. $P < 0.05$ was used as the threshold to indicate statistical significance. All the P values in present study are two-sided.

Results

In this study, 25 DNA samples including 11 from fresh-frozen cervical carcinomas and 14 from fresh-frozen HSILs were detected using the method of HPV capture combined with next generation sequencing (Table 1). RNA sequencing (RNA-seq) was applied to validate viral-human breakpoints.

Table 1
Summary of 25 clinical samples

Sample ID	Pathology	TCT	HPV types	Integration site	High Confidence Integration site[a]
17C057559	CA	NILM	16	46	9
18C042804	CA	ASCUS	16	101	10
18C059797	CA	HSIL	16	212	9
18C042806	CA	AGC	16	13	0
18C049347	CA	ASC-H	16	191	0
19C085490	CA	NILM	16	392	3
19C085491	CA	NILM	16	429	13
19C085492	CA	HSIL	16	273	11
19C085496	CA	NILM	16	85	2
19C086873	CA	HSIL	16	480	37
19C125589	CA	HSIL	16	2828	310
18C042805	HSIL	LSIL	16	14	0
18C059795	HSIL	HSIL	16	109	4
18C059796	HSIL	LSIL	16	481	25
19C085479	HSIL	HSIL	16	16	0
19C085480	HSIL	ASCUS	16	99	0
19C085481	HSIL	ASCUS	16	11	0
19C085482	HSIL	HSIL	16	2142	6
19C085483	HSIL	ASCUS	16	1811	11
19C085484	HSIL	ASCUS	16	6170	165
19C085485	HSIL	HSIL	16	3941	23
19C085486	HSIL	NILM	16	39	2
19C085487	HSIL	ASCUS	16	20	0
19C085494	HSIL	HSIL	16	311	6
19C085497	HSIL	ASCUS	16	29	0

CA: cervical cancer, HSIL: high-grade squamous intraepithelial lesion, TCT: Thinprep cytologic test, NILM: negative for intraepithelial lesion or malignancy, ASCUS: negative for intraepithelial lesion or malignancy, LSIL:low-grade squamous intraepithelial lesion, ASC-H: typical squamous cells cannot exclude HSIL, AGC: atypical glandular cells, [a] HPV-chromosomal junctions, which support reads were more than 10.

Determination of potential HPV integration sites

As described in the Bioinformatics Analysis method, if a specific position has one or more discordant read pairs mapped with one end to a human chromosome and the other to the HPV reference genome, it will be considered as a potential HPV integration site. A total of 20243 potential HPV integration sites were discovered in 25 HPV16-positive cases including 5050 integration sites for 11 cervical cancers and 15193 sites for 14 HSILs, with frequencies ranging from 11 to 6170 per sample (Table 1).

Characterization of integration breakpoints

We calculated the frequency in each viral gene or region and found that breakpoints were prone to occur in L1 and L2 instead of E2 in the cervical cancer ($P = 0.0004$ and $P = 5.15\times10^{-40}$, Fig. 1) and HSIL group ($P = 2.1\times10^{-32}$ and $P = 7.06\times10^{-13}$, Fig. 2). Unexpectedly, in contrast with reports that integrated HPV16 should retain intact oncogenes E6 and E7 with the long control region (LCR). We determined that breakpoints were less prone to occur in the LCR ($P = 2.1\times10^{-32}$ and $P = 7.06\times10^{-13}$, Figs. 1 and 2), which was probably preserved because it acted as a strong cis-activator of nearby oncogene expression, promoting the malignant transformation of host cells. In addition, we found that breakpoints were less prone to occur in the E1 ($P = 3.79\times10^{-12}$ and $P = 0.00007$, Figs. 1 and 2). These findings contradicted that in HPV integration, the disruption of the E1 or E2 gene (a negative regulator of oncogenes E6 and E7) is preferred, which may lead to the dysregulation of oncoproteins E6 and E7, thereby promoting cervical carcinogenesis.

98.17% of HPV16 gene integration sites occurred in the non-coding regions of the host gene, 42.15% of the integration sites were in the intron region of the host gene, 53.44% were integrated in the intergenic region, and only 1.83% were integrated in the exon region of the gene (Table 2). To investigate HPV integration patterns in human genome, we annotated HPV integration breakpoints in specific genomic elements. For instances, HPV16 showed a strong tendency towards intronic region ($P = 5.02\times10^{-64}$) but a subtle tendency towards intergenic region (P = 0.04). Breakpoints were less prone to occur in untranslated regions ($P = 5.02\times10^{-50}$) (Fig. 3).

Table 2
Integration sites in human genes in the 11 cervical cancers and 14 HSILs.

Host_Gene	Total	CA (N = 11)	HSIL (N = 14)	*P* Value
exonic	371	94	277	**0.001**
intronic	8533	2229	6304	
intergenic	10817	2582	8235	
UTR	522	145	377	

UTR: untranslated region, CA: cervical cancer, HSIL: high-grade squamous intraepithelial lesion. *P* values were calculated by chi-squared tests.

MACROD2 gene as HPV hot spot integration site

We focused on 646 different HPV-chromosomal junctions (inter- or intra-genic), which support reads were more than 10. The most frequent integration site was in the MACROD2 gene (n = 7) followed by the BCAS3 (n = 2), DLG2 (n = 2), N4BP3 (n = 2), PRIM2 (n = 2) and RIN2 (n = 2)(Table 3). Patients with HPV integration sites into the MACROD2 gene (introns 2, 4, 5, 6, 8 and 9). Consistently, *Kamal et al.* found that patients with HPV integration sites into the MACROD2 gene (introns 5, 6 and 7)[14]. Combined with previous research, it can be found that breakpoints were mainly occurred in the introns 5 of MACROD2 gene (Fig. 4).

Table 3
Detailed information on HPV hot spot integration site detected from virus capture sequencing data.

Sample	Group	Transcript 1	Position 1	CytoBand	Transcript 2	Host_Gene	Region	Position 2
18C042804	CA	L1	5822	20p12.1	chr20	MACROD2	intronic 5	14857764
	CA	E1	1718	20p12.1	chr20	MACROD2	intronic 5	15063527
	CA	E1	1722	20p12.1	chr20	MACROD2	intronic 5	15063531
	CA	E1	1722	20p12.1	chr20	MACROD2	intronic 5	15063533
	CA	L1	5822	20p12.1	chr20	MACROD2	intronic 5	14857760
	CA	L1	5822	20p12.1	chr20	MACROD2	intronic 5	14857763
	CA	L1	5822	20p12.1	chr20	MACROD2	intronic 5	14857765
19C085482	HSIL	E6	138–272	20p12.1	chr20	MACROD2	intronic 7	15462911
19C085483	HSIL	LCR	7678–7812	20p12.1	chr20	MACROD2	intronic 5	15105141
	HSIL	LCR	7693–7827	20p12.1	chr20	MACROD2	intronic 5	14836445
	HSIL	L2	5369–5503	20p12.1	chr20	MACROD2	intronic 8	15502700
19C085485	HSIL	E6	2-136	20p12.1	chr20	MACROD2	intronic 6	15239626
	HSIL	E1	2238–2372	20p12.1	chr20	MACROD2	intronic 2	14012753
	HSIL	E1	2715–2849	20p12.1	chr20	MACROD2	intronic 7	15442160
	HSIL	L2	4628–4762	20p12.1	chr20	MACROD2	intronic 9	15874197
19C085486	HSIL	E2	3228–3362	20p12.1	chr20	MACROD2	intronic 4	14548280
19C085492	CA	LCR	7248–7382	20p12.1	chr20	MACROD2	intronic 5	15167758
19C085494	HSIL	L1	6909–7043	20p12.1	chr20	MACROD2	intronic 5	15118669
19C086873	CA	E1	2368–2502	20p12.1	chr20	MACROD2	intronic 5	14817896
19C085484	HSIL	E5	3866	17q23.2	chr17	BCAS3	intronic	60751193
19C085485	HSIL	L1	6709–6843	17q23.2	chr17	BCAS3	intronic	60704149
19C125589	CA	E2	3258	17q23.2	chr17	BCAS3	intronic	61146950
19C085484	HSIL	L1	6511	20q13.13	chr20	BCAS3	intronic	50873654
19C085482	HSIL	L2	5187–5321	11q14.1	chr11	DLG2	intronic	84217270

CA: cervical cancer, HSIL: high-grade squamous intraepithelial lesion.

Sample	Group	Transcript 1	Position 1	CytoBand	Transcript 2	Host_Gene	Region	Position 2
19C085483	HSIL	L2	5550–5684	11q14.1	chr11	DLG2	intronic	83962461
19C085484	HSIL	E6	234	11q14.1	chr11	DLG2	intronic	84168220
19C085485	HSIL	L2	4943	11q14.1	chr11	DLG2	intronic	84298707
19C085485	HSIL	E7	724–858	11q14.1	chr11	DLG2	intronic	83672460
19C085485	HSIL	L2	4644–4778	11q14.1	chr11	DLG2	intronic	83947854
19C085485	HSIL	L2	5398–5532	11q14.1	chr11	DLG2	intronic	84215748
19C085485	HSIL	E2	2953–3087	11q14.1	chr11	DLG2	intronic	85518222
19C085485	HSIL	E6	20–154	11q14.1	chr11	DLG2	intronic	84615729
19C086873	CA	L1	6654–6788	11q14.1	chr11	DLG2	intronic	85603726
19C086873	CA	E2	2944–3078	11q14.1	chr11	DLG2	intronic	84874625
18C059797	CA	E2	2908	5q35.3	chr5	N4BP3	UTR5	1.78E + 08
18C059797	CA	L2	4872	5q35.3	chr5	N4BP3	UTR5	1.78E + 08
19C085483	HSIL	E2	2908	5q35.3	chr5	N4BP3	UTR5	1.78E + 08
19C085483	HSIL	L2	4872	5q35.3	chr5	N4BP3	UTR5	1.78E + 08
19C085484	HSIL	L2	4953	6p11.2	chr6	PRIM2	intronic	57315456
19C085484	HSIL	L1	6196–6330	6p11.2	chr6	PRIM2	intronic	57472028
19C125589	CA	L1	6322	6p11.2	chr6	PRIM2	intronic	57488118
19C085484	HSIL	L1	5929	6p11.2	chr6	PRIM2;GUSBP4	intergenic	57863258
19C085485	HSIL	E2	3090–3224	6p11.2	chr6	PRIM2;GUSBP4	intergenic	57741468
19C085483	HSIL	E1	2271–2405	20p11.23	chr20	RIN2	intronic	19916317
19C085484	HSIL	E6	528	20p11.23	chr20	RIN2	intronic	19965616
19C125589	CA	L2	4503	20p11.23	chr20	RIN2	intronic	19987241

CA: cervical cancer, HSIL: high-grade squamous intraepithelial lesion.

To validate HPV integration breakpoints detected by HPV probes and to investigate whether HPV continues to transcribe viral genes after integrating into the host genome, we performed RNA–seq on 3 samples. The number of integration sites

at the RNA level (n = 11) is significantly lower than that at the DNA level (n = 19). Comparison of RNA and DNA breakpoints in the HPV genome revealed two patterns of breakpoint distribution in the same samples. Our data suggested the possibility that HPV integration may first occur in the E1/L1 genes (Table 4) and RNA splicing may switch the breakpoint positions to the E1/L2/LCR genes. The HPV integration site still occurs in the intron region of the gene, and the RNA retains the intron region of the host gene (Table 4).

Table 4
Characteristic of HPV Integration in the Genome and Transcriptome on 3 samples.

Style	Sample	Transcript 1	Position 1	Transcript 2	Host_Gene	Region	Position 2
DNA	18C042804	L1	5822	chr20	MACROD2	intronic 5	14857764
		E1	1718	chr20	MACROD2	intronic 5	15063527
		E1	1722	chr20	MACROD2	intronic 5	15063531
		E1	1722	chr20	MACROD2	intronic 5	15063533
		L1	5822	chr20	MACROD2	intronic 5	14857760
		L1	5822	chr20	MACROD2	intronic 5	14857763
		L1	5822	chr20	MACROD2	intronic 5	14857765
	18C049347	NONE					
	17C057559	L1	6187	chr16	CDH13	intronic 1	82801743
		E2	3609	chr16	CDH13	intronic 2	82957438
		L1	6190	chr16	CDH13	intronic 1	82801742
RNA	18C042804	*LCR*	7690–7732	chr20	MACROD2	intronic 5	14847651
		E7	579–726	chr20	MACROD2	intronic 5	15063527
		LCR	7652–7829	chr20	MACROD2	intronic 6	15245852
		LCR	7147–7862	chr20	MACROD2	intronic 15	16030471
	18C049347	*LCR*	7702–7806	chr3	*TP63*	intronic 1	189713170
		E1	2492–2623	chr10	—	—	105420740
	17C057559	*E1*	2491–2648	chr13	*LINC00393*	—	73593495
		L2	4771	chr13	*LINC00393*	—	73592815
		E1	7651–7795	chr13	*LINC00393*	—	73592816
		LCR	7208–7856	chr16	*CDH13*	intronic 1	82797552
		L2	5104–5218	chr16	*CDH13*	intronic 1	82801704

Discussion

Analysis of cervical squamous cell carcinoma shows that human papillomavirus (HPV) integration occurs in more than 80% of cervical cancers[15]. Many studies have compared the human genomic regions associated with HPV integration sites to elucidate the mechanisms that might promote integration and carcinogenesis[16]. Integration of HPV DNA occurs in all human chromosomes; however, integration sites are often found within or in close proximity to common fragile sites[17]. A series of hotspots genes integrated by HPV had been found in the recent study[10]. Despite increased attention

on HPV integration hotspots, the characteristic of HPV integration and the relationship between HPV integration and cervical cancer remained elusive.

The HPV breakpoints could occur in any part of the viral genome, perhaps enabling the virus to adapt to the changing environment during carcinogenesis[18]. It is reported that the hinge region of the HPV-E2 gene was the most common deletion or breakage site when the HPV DNA integrates into the host genome[19]. The disruption of E2 blocks the viral replication that resulted in the aberrant viral gene expression, loss of control on E6 and E7 proteins, and ultimately leading to CC progression[20]. We calculated the frequency in each viral gene or region and found that breakpoints were prone to occur in L1 and L2 instead of E2 in the cervical cancer (P = 0.0004 and P = 5.15×10−40) and HSIL group (P = 2.1×10−32 and P = 7.06×10−13). The HPV16 L1 protein activates innate immunity through the type I interferon pathway and exhibits an efficient anti-cancer effect when cooperating with immune checkpoint blockade therapy[21]. Furthermore, the L1 coding sequences of the immunogenic surface loops are distinctively poorly conserved due to selective pressures for mutagenesis and immune evasion[22]. Therefore, we speculate that HPV integration leads to immune escape by destroying HPV L1.

In this study, the 20243 potential HPV integration sites in 25 HPV16-positive cases were used to carry out the bioinformatic analysis. We found that HPV16 showed a strong tendency towards intronic region ($P = 5.02\times10^{-64}$) but a subtle tendency towards intergenic region (P = 0.04). Our result was consistent with previous study showing that breakpoints of DNA samples were significantly prone to the region of INTRON (P < 0.01, Chi-squared test)[11]. Moreover, *Li W et al* found that the breakpoints are significantly enriched in the INTRON and PROMOTER regions[23]. Therefore, it might be directly related to the disruption and alteration of gene function.

We focused on 646 different HPV-chromosomal junctions, The most frequent integration site was in the MACROD2 gene (introns 2, 4, 5, 6, 8 and 9). Consistently, *Kamal et al.* found that patients with HPV integration sites into the MACROD2 gene (introns 5, 6 and 7)[14]. *Juliette Mainguené et al.* reported that the third HPV integration hotspot is MACROD2 (4.1%) in head and neck squamous cell carcinoma, with two patients displaying intragenic HPV integration[24]. MACROD2 is a protein-coding gene located at a fragile site on human chromosome 20. The MACROD2 protein is a deacetylase involved in the removal of ADP-ribose from mono-ADP-ribosylated; it has a key role in DNA repair[25]. MACROD2 deficiency promotes tumor growth and metastasis by activating GSK-3β/β-catenin signaling in Hepatocellular carcinoma[26]. MACROD2 is a caretaker tumour suppressor gene. MACROD2 loss causes repression of PARP1 activity, impairing DNA repair[27, 28]. In breast cancer, MACROD2 overexpression mediates estrogen-independent growth and tamoxifen resistance[29]. The protein data bank showed that the functional domain of MACROD2 protein was located at amino acids 59 ~ 240 and mainly interacted with PARP1. The HPV integration sites were in this functional domain, which alter DNA repair and sensitivity to DNA damage thought impaired PARP1 activity resulting in chromosome instability.

For many years, HPV oncogenic potential was only attributed to the viral oncoproteins E6 and E7, but recent studies highlights that HPV integration is an oncogenic event. In our study, a large portion of HPV integration sites in DNA samples was located on the no-coding region (Intron, Intergenic). It might suggest that HPV integration could directly trigger the abnormal transcription and these functions of novel transcript kept unclear. We described recurrent HPV integration in MACROD2 region, which in MACROD2 functional domain. MACROD2 loss alter DNA repair and sensitivity to DNA damage thought impaired PARP1 activity resulting in chromosome instability.

Declarations

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Author contributions JWZ, WZ, LQWconceived and designed the experiments; JWZ and SJK performed the experiments, Funding acquisition; JWZ and XLW contributed reagents/materials/analysis tools; JWZ and WZ wrote the paper, JLC and JQH revised the paper. All authors read and approved the final manuscript.

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Availability of data and materials The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The study was approved by our institutional ethical review board. Informed consent had been obtained from the reported patients.

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Figures

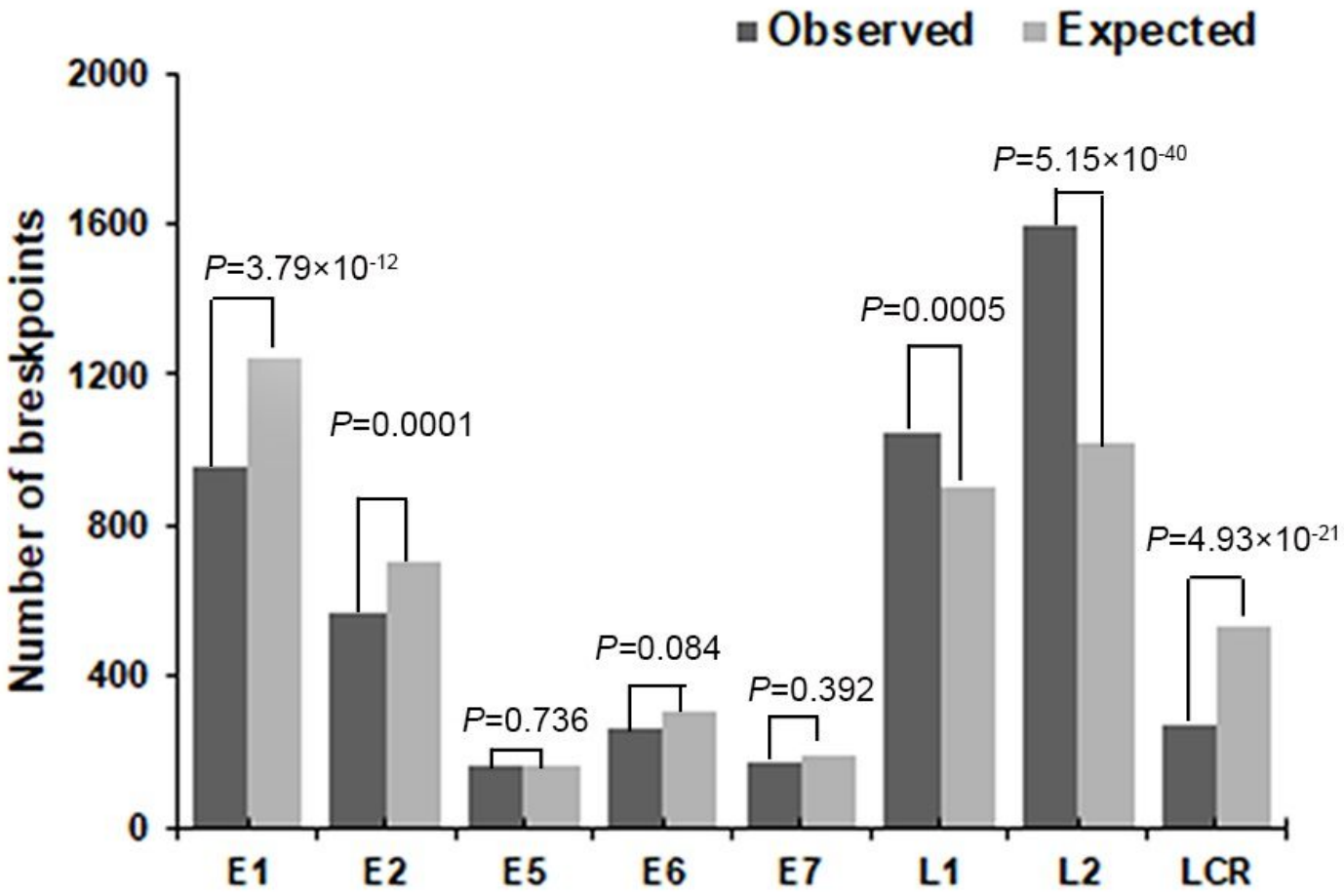

Figure 1

Comparison of the observed (black) and expected (gray) numbers of breakpoints in the cervical cancer group. *P* values were calculated by chi-squared tests.

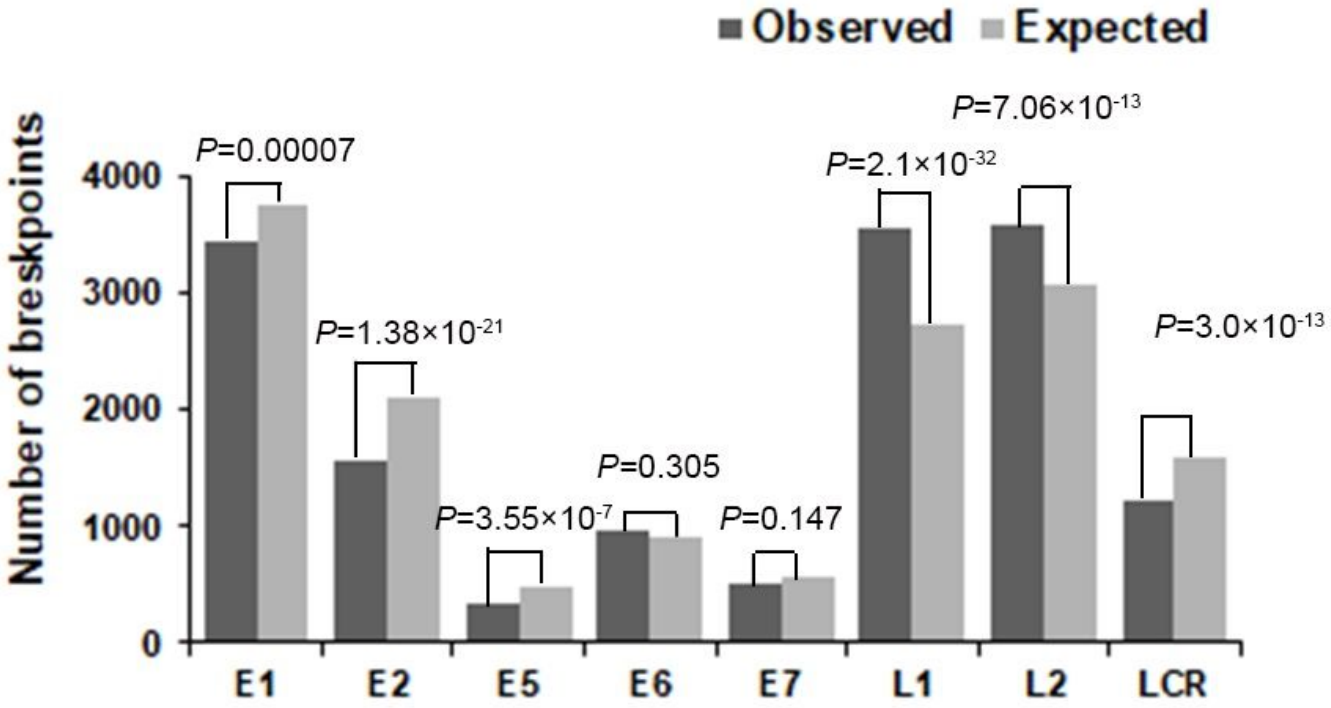

Figure 2

Comparison of the observed (black) and expected (gray) numbers of breakpoints in the HSIL group. *P* values were calculated by chi-squared tests.

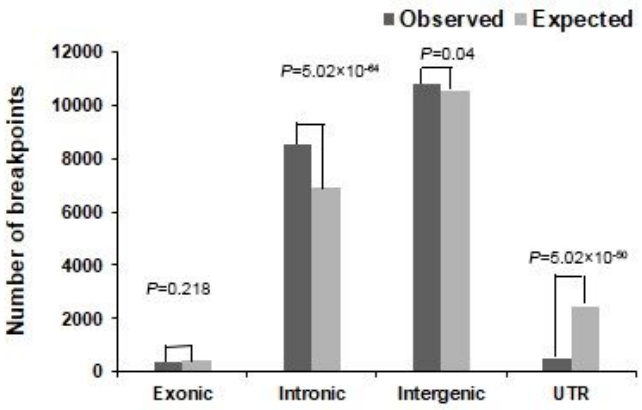

Figure 3

Distribution of integration breakpoints in human genetic elements by HPV 16 in 25 samples. *P* values were calculated by chi-squared tests.

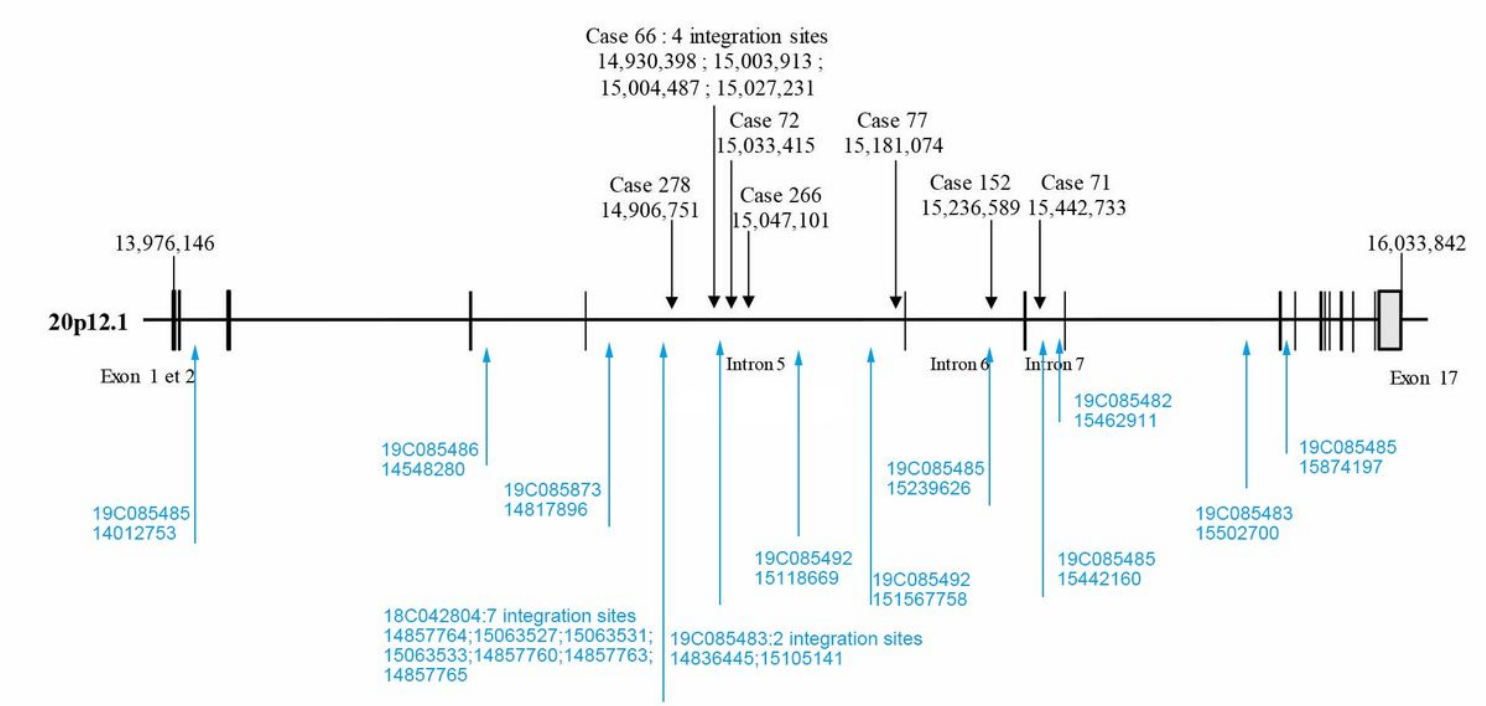

Figure 4

HPV integration sites in the MACROD2 gene (GRCh37 / hg19). The lower part of HPV integration sites(blue) were our research data, and the upper part (black) was from previous research results[14].