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REVIEW

Retinoic acid receptor beta promoter methylation and risk of cervical cancer

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Abstract

Cervical cancer is one of the leading causes of death in women worldwide, particularly in developing countries. Human papillomavirus has been reported as one of the key etiologic factors in cervical carcinoma. Likewise, epigenetic aberrations have ability to regulate cancer pathogenesis and progression. Recent research suggested that methylation has been detected already at precancerous stages, which methylation markers may have significant value in cervical cancer screening. The retinoic acid receptor beta (*RARβ*) gene, a potential tumor suppressor gene, is usually expressed in normal epithelial tissue. Methylation of CpG islands in the promoter region of the *RARβ* gene has been found to be associated with the development of cervical cancer. To investigate whether *RARβ* methylation is a potential biomarker that predicts the progression of invasive cancer, we reviewed 14 previously published articles related to *RARβ* methylation. The majority of them demonstrated that the frequency of *RARβ* promoter methylation was significantly correlated with the severity of cervical epithelium abnormalities. However, methylation of a single gene may not represent the best approach for predicting disease prognosis. Analyzing combinations of aberrant methylation of multiple genes may increase the sensitivity, and thus this approach may serve as a better tool for predicting disease prognosis.

Key words: Methylation; Cervical cancer; Retinoic acid receptor beta; Human papillomavirus; Risk correlation; Promoter

Core tip: The frequency of retinoic acid receptor beta promoter methylation was significantly correlated with the severity of cervical epithelium abnormalities. However, a single gene may not represent the best approach for predicting disease prognosis. Thus, combinations of aberrant methylation of multiple genes may as a better tool for predicting disease.

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INTRODUCTION

Cervical cancer is the leading cause of death in women worldwide. The prevalence is high in women in low- to middle-income countries[1]. In 2012, approximately 522000 women globally were diagnosed with cervical cancer, and the mortality rate due to cervical cancer was reported to be 266,000 cases/year[2]. The highest incidence occurred in sub-Saharan Africa while in Asia, cervical cancer remains the third most common cancer (after breast and lung cancer), with an estimated 285000 new cases and 144000 deaths in 2012[3]. The age-standardized incidence rates (ASRs) of cervical cancer estimated by GLOBOSCAN in 2012 indicated that the ASR is higher in less developed compared to more developed regions[4]. In Thailand, the age group with the highest incidence is 45-70 years[5].

Several studies had found that cervical cancer is preceded by a pre-invasive stage, in which abnormal cells are confined to the cervical epithelium. The pre-invasive stage is also known as cervical intraepithelial neoplasia (CIN). The 2014 Bethesda System categorizes squamous epithelial cell abnormalities as atypical squamous cell of undetermined significance (AS-CUS); low-grade squamous intraepithelial lesion (LSIL), which was previously known as CIN Ⅰ; high-grade squamous intraepithelial lesion (HSIL), which was previously known as CIN Ⅱ and Ⅲ; or squamous cell carcinoma (SCC)[6]. SCC represents > 80% of cervical cancers, while adenocarcinoma (AC) accounts for the rest.

The standard method for screening for early-stage cervical neoplasia is cytological morphologic assessment of cervical scrapings. The sensitivity of the conventional Pap smear for identifying CIN Ⅱ+ is 55.2%, while the sensitivity of liquid-based cytology is 57.1%[7]. High-risk human papillomavirus (HPV) DNA testing in combination with the conventional Pap smear increases the sensitivity. Furthermore, biomarkers of oncogenic progression would improve the accuracy of cancer progression predictions. Epigenetic biomarkers may help to fulfil this role, and they have the additional benefit predicting the stage of cervical carcinogenesis progression[8].

GENOME OF HPV

HPV is a small, non-enveloped and circular double-stranded DNA virus with a genome of approximately 8 kb in length[9]. The HPV genome comprises eight protein-coding genes and a noncoding region that is referred to as the regulatory long control region[10]. Only one strand of the DNA carries the protein-coding sequence[11]. Regarding the protein-coding genes, the genes are designated as early (E) or late (L) to indicate when the proteins are expressed in the viral life cycle[12]. The eight protein-coding gene consist of E1, E2, E4, E5, E6, E7, L1 and L2[9]. E1 and E2 are highly conserved and involved in viral DNA replication[13-15]. L1 and L2, which both have a high degree of sequence variation, encode for viral packaging proteins[16]. E4 releases the viral particle from the epithelial cells[17]. E6 and E7 are viral oncogenes that are involved in the integration of the HPV genome into the host genome[18]. There are more than 130 genotypes of HPV, which are categorized based on sequence variation in their L1 region[19]. Of the 130 genotypes, at least 40 genotypes infect the genital areas of humans *via* sexual transmission. HPV can also be classified into cutaneous or mucosal types[12]. The mucosal type can be subdivided into high-, intermediate-, or low-risk types[20].

HPV AND CERVICAL CANCER

The most important risk factor for cervical cancer is HPV infection, which has been found in 90.7% of cervical cancer patients worldwide[21]. HPV infection is a sexually transmitted disease. It has been estimated that more than 80% of sexually active women become infected with HPV, while more than 50% of young women become infected after they first have sexual intercourse[22]. The oncogenic potential of HPV depends on the genotype. HPV 16 and 18 are the most common types associated with invasive cervical cancer[23]. Other HPV genotypes have been found to be related to cancer, but their oncogenic risk differs among the various populations, geographic regions, and age groups.

At the country level, collecting baseline data on the local burden of specific HPV genotypes related to cervical cancer is important. This information can impact the local HPV vaccination policies. A meta-analysis revealed that HPV 16, 18, 31, 33, 45, 52, and 58 are responsible for more than 90% of cervical cancers worldwide[20]. These genotypes represent the baseline genotypes to include in a vaccine targeting the genotypes circulating in the population[4]. The current HPV vaccines were developed to prevent HPV infection, and thus prevent cervical carcinoma. HPV vaccines have been implemented in routine vaccination programs in several developed and developing countries worldwide[24]. To

date, there have been three HPV vaccines in clinical use: Bivalent, quadrivalent, and nanovalent vaccines[25].

Other independent risk factors such as immunosuppression, individual lifestyle, and smoking have been found to be associated with the development of HPV-related cervical cancer[21,26]. Most HPV infection is transient, and clearance of the virus can occur spontaneously over a 3-year period[27]. However, in some cases, persistent infection can result in cervical cancer development. The transition from dysplasia to invasive carcinoma may take several years to decades to develop. HPV initially infects the basal layers of the epithelium through micro-wounds. The virus begins to replicate, and when infected daughter cells migrate to the upper layers of the epithelium, the viral late genes are activated, and viral DNA is packaged into capsids. Progeny virions are released to re-initiate infection, which can result in persistent and/or asymptomatic infection[28]. The integration of HPV into the host genome can lead to carcinogenic transformation. Certain regions of the human genome are favored for viral DNA insertion such as fragile sites, rupture points, translocation points, and transcriptionally active regions[29]. Moreover, the virus can induce epigenetic modification of viral and cellular genes, which affect their expression, leading to malignant cell transformation[30,31].

HOST GENETIC FACTORS AND CERVICAL CANCER

Diverse immunogenetic associations with HPV infection, persistence, and transformation have been extensively investigated. Recent studies have looked at multiple genes in various populations with different environment interactions[32]. HPV infection alone might not be sufficient for the development of cervical carcinoma, and certain antigen-processing machinery (APM) and single-nucleotide polymorphisms (SNPs) may lead to a smaller immunogenic peptide repertoire for presentation to local immune cells. This can result in further attenuation of cytokine and receptor expression, which leads to an ineffective overall immune response and progression to carcinoma[33]. The Genome-Wide Association Study (GWAS) for polymorphisms of host immune response genes showed that variation in several genes contributes to different risks of cervical cancer. The integrative approach, which is also known as systems biology, could help explain the complexity of host–virus interactions and provide a better understanding that may eventually lead to personalized prevention, diagnosis, and treatment[34-36].

The detection of methylated genes in cervical specmens is a feasible technique and represents a potential source of biomarkers that are of relevance to carcinogenesis. In particular, there are methylation markers that, among HPV-infected women, indicate the presence of CIN Ⅱ+ and risk of cancer[37].

High expression levels of certain oncoproteins in cervical cells have been found to be associated with cervical carcinoma. One study found a strong correlation between centromere protein H (CENP-H) expression and cervical carcinoma in a Chinese population[38]. Another study found that expression of the B-cell-specific Moloney leukemia virus insert site 1 (Bmi-1), P16, and CD44v6 (a CD44 variant) were significantly higher in cervical carcinoma tissues compared with precancerous lesions and normal tissues[39]. In addition, abnormalities in the phosphatidylinositide 3-kinase (PI3K) pathway induced by mutations in PI3K catalytic subunit α (PIK3CA) were associated with shorter survival in cervical cancer patients[40]. Recently, deep sequencing of somatic mutations has identified several novel mutations in carcinoma cells, including E322K in the mitogen-activated protein kinase 1 (*MAPK1*) gene, inactivating mutations in the major histocompatibility complex, class I, B (HLA-B) gene, and mutations in F-box and WD repeat domain containing 7 (*FBXW7*), tumor protein p53 (*TP53*), and Erb-B2 receptor tyrosine kinase 2 (*ERBB2*)[41].

EPIGENETIC MECHANISMS AND RISK OF CANCER DEVELOPMENT

Recent studies also investigated epigenetic mechanisms related to HPV infection, including methylation of the host and viral genes, and chromatin modification in host cells[42]. Epigenetic mechanisms affect gene regulation without changing the genetic sequences, and these mechanisms have been increasingly found to be associated with cancer development[43]. The main epigenetic mechanism is methylation patterning, which occurs to various extents in different DNA and proteins. DNA methylation is a mechanism of gene regulation that typically occurs in CpG dinucleotide contexts, resulting in genomic instability. Methylation of heterochromatin and promoter regions is associated with decreased gene transcription. Several studies have found that DNA methylation frequently occurs in cervical cells but rarely in normal cells, suggesting that their methylation is highly related to the severity of cervical neoplasia[44]. Several markers have been evaluated extensively in studies involving women with precancerous and cancerous cervical lesions[44-46]. Epigenetic methylation in the promoter region of several tumor suppressor genes (TSGs) has been detected in precancerous cervical cells[47,48]. Genes that were found to be significantly associated with promoter methylation were RASSF1A and MGMT (involved in DNA repair), CDKN2A (involved in cell cycle control), PYCARD (involved in apoptosis), and APC and SFRP1 (involved in Wnt signaling)[49].

One striking conclusion of previous studies was that methylation frequencies for the same gene vary widely between studies. It was difficult to identify highly consistent results for most genes even when restricting analyses to studies of similar size or those that used common specimen sources or similar assays.

This suggests that the frequency of certain methylation markers may also vary for reasons related to differences in populations, specific features of assay protocols, chance, or other unidentified factors. The most important prerequisite for a potential biomarker is that it must be reliable in its measurement. There is a possibility that the wide range of frequencies reported for some genes (in contrast to the more consistent measurement of a few other genes in similar studies) could be related to unreliable assays for these specific genes rather than biological variation. Another prerequisite for a good biomarker is that it has high sensitivity and high specificity for disease detection, resulting in a high positive predictive value. Several studies have proposed the use of methylated gene panels in order to obtain optimal assessment performance for cervical cancer screening[47,50].

Retinoic acid (RA) is an essential regulator of normal epithelial cell differentiation. The effect of RA is mediated by two types of nuclear receptors, the retinoic-acid receptor (RAR) family and retinoid-X receptor (RXR) family. Both of these receptor families have three members (alpha, beta, and gamma), which are encoded by distinct genes in vertebrates. The retinoic acid receptor beta (*RARβ*) gene encodes a nuclear receptor that binds RA and mediates cellular signaling. It is important during differentiation of stratified squamous epithelium, including cervical epithelium. It is considered to be a potential TSG. The *RARβ* gene is usually expressed in normal epithelial tissue. The direct roles of the RARβ protein include regulating gene expression and differentiation, immune modulation, and inducing apoptosis. Previous studies revealed that the *RARβ* gene is downregulated in high-grade lesions[51]. *RARβ* gene silencing was observed in carcinoma cells[52]. Recent research suggested that the RARβ protein can suppress cervical carcinogenesis and may play a role in the early development of cancer[51]. CpG methylation of the 5' region of the *RARβ* gene contributes to gene silencing, and this methylation is associated with increased grades of SIL and invasive cervical cancer. Many studies have revealed that methylation of CpG islands in the promoter region of the *RARβ* gene induces repression of *RARβ* expression in several epithelial carcinomas, including cervical cancer[53-55].

The risk of cervical cancer due to *RARβ* methylation remains inconsistent across different studies[51,52,56]. Therefore, we reviewed previously published articles and summarized the relationship between *RARβ* promoter methylation and cervical cancer (Table 1).

Among the 14 articles reviewed, the majority of them (11/14) demonstrated that the frequency of *RARβ* promoter methylation was significantly correlated with severity of cervical epithelium abnormalities. Three studies did not concur with this finding. The first study was conducted in 2003 with a small sample size and no cancer patients were involved[37]. The other two studies were conducted in 2010 and 2015. Both studies found that normal tissue also had *RARβ* promoter methylation, which made it a poor predictor of progression to severe disease[62,64]. However, one of the two studies also investigated the level of methylation using quantitative methylation-specific PCR and found that although normal cells were methylated, the level of methylation increased in LSIL, HSIL, and invasive cancer tissue[62].

In addition, both Narayan *et al*[56] and Choi *et al*[60] found that *RARβ* promoter methylation was associated with cervical cancer prognosis. Narayan *et al*[56] found that 80% of the patients with *RARβ* methylation either died of cancer or only partly responded to treatment, while Choi *et al*[60] found that absence or reduction of RARβ protein expression was associated with a higher level of SCC antigen (P = 0.04) and more frequent lymph node metastasis (P = 0.023).

A study of the frequency of *RARβ* promoter methylation in urine and cervical samples from Senegalese women and cervical epithelial cell abnormalities found that methylation was significantly greater in abnormal specimens (and the results from the urine samples correlated with the results from the cervical swab samples)[58,65]. Another study by Zhang *et al*[52] compared the frequency of methylation with *RARβ* mRNA expression. The authors found that in normal cervical cells, the *RARβ* gene was highly expressed. In contrast, among 17 samples from patients with invasive cervical carcinoma, *RARβ2* expression was completely repressed in 13 samples, highly repressed in 2 samples, and moderately downregulated in 2 samples. Among the 13 samples with completely repressed *RARβ2* expression, the *RARβ* promoter region was methylated in 9 samples and unmethylated in 4 samples. The authors then further investigated the silencing mechanism and discovered that apart from methylation, repressive histone modifications also played a role in gene silencing, which could contribute to the development of cervical carcinoma.

Four studies performed a quantitative assessment of methylation. The first study was conducted in 2006 by Wisman *et al*[59], who found that the *RARβ2* promoter was more methylated in cervical cancer than in control tissue. Four years later, Kim *et al*[61] found that the *RARβ* methylation level in normal tissue was 1.59% ± 3.51% whereas, in HSIL and SCC, it was 21.93% ± 20.10% and 19.06% ± 19.39%, respectively. The third study, by Yang *et al*[62], also highlighted that although the percentage of methylated samples was very high in normal tissue, the level of methylation correlated with disease severity. The last study was conducted by Sun *et al*[51] in 2015. They found that among 250 cervical samples from healthy individuals and patients with various stages of cervical epithelium abnormalities, the percentage of methylation in patients showed that 68.8% had no *RARβ* promoter methylation, 26.4% had 0%-5% methylation, and 4.8% had 5%-25% methylation. No samples had methylation levels above 25%.

In addition, two studies performed immunohistochemistry staining of the RARβ protein in cervical cells. Narayan *et al*[56] found that in the LSIL group, 11% had

Table 1 The summary of the articles that investigated the methylation of *RARβ* gene in tumor tissue from women diagnosed with squamous intraepithelial lesion and cervical cancer

Ref.	Year of publication	Nationality of participants	Sample size	Source of samples	Lab technique	*RARβ* methylation results
Virmani *et al*[57]	2001	American	Normal/LSIL = 37	Normal/LSIL/HSIL from liquid-based cytology specimen	MSP	*RARβ* methylation positive in
			HSIL = 17 ICC = 19	ICC from biopsy tissue		Normal/LSIL = 11% HSIL = 29% ICC = 26%
Narayan *et al*[56]	2003	Colombians	Normal = 8	Normal = cells from cervical swab LSIL/HSIL = formalin-fixed and paraffin-embedded cervical tissues	MSP	*RARβ* methylation positive in
		German	LSIL = 9			Normal = 0% SCC/AC = 29.3%
		American	HSIL = 30	SCC/AC = tumor biopsies	Immuno-histochemistry of *RARβ* protein	Immunohistochemistry
			SCC = 77 AC = 5			LSIL; 11% showed low expression HSIL; 60% showed complete lack of expression
Gustafson *et al*[37]	2004	American	Normal = 11	Liquid-based cytology specimen	Nested MSP	*RARβ* methylation positive in
			LSIL = 17 HSIL = 11			Normal = 0% LSIL = 0% HSIL = 9.1%
Feng *et al*[58]	2005	Senegalese	Normal/ASCUS = 142	Exfoliated cervical cells and tissue biopsy	MSP	*RARβ* methylation positive in
			CIN Ⅰ = 39 CIN Ⅱ = 23 CIN Ⅲ = 23 ICC = 92			Normal/ASCUS = 3.2% CIN Ⅰ = 0% CIN Ⅱ = 0% CIN Ⅲ = 15.8% ICC = 38.2%
Wisman *et al*[59]	2006	Dutch	Normal = 19	Cervical scraping	QMSP	The percentage of *RARβ* methylation level above control ratio were detected in Normal = 0% SCC = 15% AC = 25%
			SCC = 20 AC = 8			
Choi *et al*[60]	2007	Korean	Normal = 37	Normal cells were from hysterectomy due to myoma	MSP	*RARβ* methylation positive in
			SCC = 37	Cancer cells were from tissue after surgery	Immuno-histochemistry of *RARβ* protein	Normal = 0% SCC = 41%
						Immunostaining normal = strong staining SCC = 43% absent staining
Zhang *et al*[52]	2007	Japanese and Chinese	Normal = 6	Cervical tissue by biopsy or surgery	Real-time PCR for *RARβ* mRNA	*RARβ* expression level among normal cells: All were highly expressed
			ICC = 17			RARb2 expression level among cancer cells:
					Semi-nested MSP	13/17: Completely repressed
						2/17: Highly repressed 2/17: Moderately down-regulated Among 13 samples with completely repressed mRNA expression 9 promoter methylated, 4 unmethylated
Flatley *et al*[2]	2009	English	Normal = 58	Exfoliated cervical cells and cervical biopsy	Nested MSP	*RARβ* methylation positive in
			CIN Ⅰ = 68 CIN Ⅱ = 56 CIN Ⅲ = 76 ICC = 50			Normal = 6.5% CIN Ⅰ = 42.6% CIN Ⅱ = 6.3% CIN III = 0% ICC = 15.9%
Kim *et al*[54]	2010	Korean	Normal = 41	Liquid based cytology specimen	Multiplex nested MSP	*RARβ* methylation positive in
			LSIL = 32 HSIL = 67 SCC = 69			Normal = 4.9% LSIL = 15.6% HSIL = 46.3% SCC = 53.6%
Kim *et al*[61]	2010	Korean	Normal = 28	Liquid based cytology specimen	Multiplex QMSP	*RARβ* methylation level

			LSIL = 26			Normal = 1.59+3.51% LSIL = 3.67+9.09%
			HSIL = 45 SCC = 63			HSIL = 21.93+20.10% SCC = 19.06+19.39%
Yang *et al*[62]	2010	Dutch	Normal = 20	Biopsy tissue	QMSP	*RARβ* methylation positive (from tissue) in
			LSIL = 20			Normal = 85% LSIL = 65%
			HSIL = 20	Cervical scraping only available in subset of samples		HSIL = 75% SCC = 85% AC = 85%
			SCC = 40			*RARβ* methylation positive (from scraping) in
			AC = 20			Normal = 44% LSIL = 37.5% HSIL = 55.6% SCC = 83.8% AC = 100% The median methylation level increased significantly with the severity of lesion ($P < 0.05$)
Pathak *et al*[63]	2012	Indian	Normal = 35	Normal cells from hysterectomy SIL from excision ICC from tissue biopsy	MSP	*RARβ* methylation positive in
			SIL = 27			Normal = 11.4% SIL = 55.5% ICC = 57.8%
			ICC = 38			
Milutin Gašperov *et al*[64]	2015	Croatian	Normal = 40	Cervical scraping	MSP	*RARβ* methylation positive in
			CIN Ⅰ = 40			Normal = 62.5%
			CIN Ⅱ = 40			CIN Ⅰ = 35%
			CIN Ⅲ = 42			CIN Ⅱ = 61.5%
			SCC = 8 AC = 3			CIN Ⅲ = 61.9% SCC/AC = 90%
Sun *et al*[51]	2015	Chinese	Normal = 48	Liquid based cytology specimen	Methylation specific high resolution melting analysis (Quantitative)	*RARβ* methylation positive in
			CIN Ⅰ = 54			Normal = 31.3% CIN Ⅰ = 35.2%
			CIN Ⅱ = 47			CIN Ⅱ and Ⅲ = 28.2% SCC = 33.3%
			CIN Ⅲ = 56			*RARβ* methylation level: none = 68.8%
			SCC = 45			0-5% methylation = 26.4% 5-25% = 4.8%

CIN: Cervical intraepithelial neoplasia; SIL: Squamous intraepithelial lesion; LSIL: Low-grade squamous intraepithelial lesion; HSIL: High-grade squamous intraepithelial lesion; SCC: Squamous cell carcinoma of the cervix; AC: Adenocarcinoma of cervix; ICC: Invasive cervical cancer; MSP: Methylation-Specific Polymerase Chain Reaction; QMSP: Quantitative methylation-specific polymerase chain reaction; ASCUS: Atypical squamous cells of undetermined.

low RARβ expression whereas, in the HSIL group, 60% had a complete lack of RARβ expression. This finding suggested that the downregulation of the *RARβ* gene occurs early in the development of cervical carcinoma[56]. The second study was carried out by Choi *et al*[60], who discovered that all normal tissues highly expressed the RARβ protein, whereas no staining was detected in 43% of the SCC tissues.

Almost of cancer cell lines and primary cancer tissues examined, the *RARβ2* was repressed. The repression was frequently associated with promoter methylation, which causes lack of gene expression. These results strongly support the hypothesis that promoter methylation is the epigenetic cause of *RARβ2* repression in cervical cancers harboring methylated *RARβ2* promoters. A DNA demethylating reagent can reactivate gene expression by inducing drastic demethylation of the promoter in repressed cells carrying a methylated promoter[44]. This consistency between promoter demethylation and *RARβ2* derepression strongly suggests that the primary cause of *RARβ2* repression is indeed promoter methylation.

Several hypotheses have been proposed regarding the mechanisms of DNA methylation that lead to silencing of genes. In some cancer cells and tissues examined, *RARβ2* was repressed without promoter methylation. These facts indicate that although DNA methylation is the major epigenetic mechanism for gene silencing, there are other epigenetic silencing pathways independent of DNA methylation. *RARβ2* is frequently silenced in cervical cancers by one of two epigenetic mechanisms. One is DNA methylation, a well-known epigenetic mechanism leading to transcriptional silencing of genes, while the other involves the formation of repressive histone modifications near the promoter, by unknown mechanisms independent of DNA methylation. At present, the initial causes of these epigenetic changes during carcinogenesis are unclear. *RARβ2* silenced by promoter methylation can be reactivated by promoter hypomethylation. This result indicates the importance of examining promoter methylation if epigenetic modulation drugs are to be used for chemotherapy in patients with cervical cancers.

In conclusion, DNA methylation of TSGs likely contributes to the development of cancer. Although DNA

methylation of only one gene may not represent the complete process of epigenetic silencing, it has been shown to be significantly correlated with cervical cancer. Analyzing combinations of aberrant hyper- or hypo-methylation of multiple genes may increase the sensitivity of prognoses. Thus, this approach may serve as a better tool for predicting disease progression. Risk factors should also be further characterized to better understand the pathogenesis of cervical carcinoma.

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ORIGINAL ARTICLE

Basic Study

Identification of various cell culture models for the study of Zika virus

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Abstract

AIM

To identify cell culture models supportive for Zika virus (ZIKV) replication.

METHODS

Various human and non-human cell lines were infected with a defined amount of ZIKV Polynesia strain. Cells were analyzed 48 h post infection for the amount of intracellular and extracellular viral genomes and infectious viral particles by quantitative real-time PCR and virus titration assay. The extent of replication was monitored by immunofluorescence and western blot analysis by using Env and NS1 specific antibodies. Innate immunity was assayed by luciferase reporter assay and immunofluorescence analysis.

RESULTS

All investigated cell lines except CHO cells supported infection, replication and release of ZIKV. While in infected A549 and Vero cells a pronounced cytopathic effect was observed COS7, 293T and Huh7.5 cells were most resistant. Although the analyzed cell lines released comparable amounts of viral genomes to the supernatant significant differences were found for the number of infectious viral particles. The neuronal cell lines N29.1 and SH-SY5Y released 100 times less infectious viral particles than Vero-, A549- or 293T-cells. However there is no strict correlation between the amount of produced viral particles and the induction of an interferon response in the analyzed cell lines.

CONCLUSION

The investigated cell lines with their different tissue origins and diverging ZIKV susceptibility display a toolbox for ZIKV research.

Key words: Zika virus; Cell lines; Quantitative real-time PCR; Plaque assay; Interferon

Core tip: In this study ten different cell lines, human and non-human, from various tissues (*e.g.*, hepatocytes, keratinocytes and neuronal cells) were tested upon their susceptibility to Zika virus (ZIKV) infection. Except CHO cells all cells supported ZIKV life cycle, but differed in parts strongly in the intracellular and released amount of infectious viral particles. Investigating the interferon response showed no clear correlation between high and low producer cell lines.

Himmelsbach K, Hildt E. Identification of various cell culture models for the study of Zika virus. *World J Virol* 2018; 7(1): 10-20 Available from: URL: http://www.wjgnet.com/2220-3249/full/v7/i1/10.htm DOI: http://dx.doi.org/10.5501/wjv.v7.i1.10

INTRODUCTION

The Zika virus (ZIKV) is known since 1947 when it was isolated from a rhesus macaque monkey in a yellow fever research institute in the Zika forest of Uganda[1]. ZIKV has reached global attention during the epidemic in Brazil in the years 2015/2016. This mosquito-borne virus (Aedes aegypti and Aedes albopictus) was found to circulate only in East and West Africa[2] until a bigger outbreak occurred on the Yap Islands in Micronesia in the year 2007[3]. Another outbreak took place in French Polynesia in the year 2013. Here for the first time the congenital ZIKV syndrome (CZVS), microcephaly, the Guillain-Barré syndrome (GBS) and non-vectorborne transmission (mother to child, sexual, posttransfusion) was retrospectively documented[4-7]. However, the virus came into public focus in the beginning of 2016, when the WHO declared the Public *Health Emergency of International Concern* (PHEIC) since in context of the Brazil epidemic (WHO Zika Strategic Response Plan 2016) a clear correlation between ZIKV infection of pregnant women and fetal microcephaly development was observed[8,9]. This changed the attention from a side note to a headline, initiating a variety of research efforts to investigate the virus in more detail with respect to epidemiology, virus-associated pathogenesis and virus cell interaction.

ZIKV belongs to the Flaviviridae family, which is closely related to the Spondweni virus serocomplex. As member of the Flavivirus genus, ZIKV contains a single-stranded, RNA with positive polarity. The viral genome encodes a single polyprotein processed by host and viral proteases into three structural proteins - core (C) that forms the capsid, the precursor of the membrane protein (prM), and the envelope protein (E) - and into seven nonstructural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 that are responsible for the replication of the viral RNA[10]. The virus replication and morphogenesis occurs in the extranuclear compartment. In ZIKV-infected cells a massive remodeling of the endoplasmic reticulum (ER) to form membranous replication factories and a drastic reorganization of microtubules and intermediate filaments can be observed[11].

There is a variety of reports describing the infection of various primary cells or immortalized cell lines. Moreover, ZIKV was shown to replicate in various human cell types already like skin cells[12] and lung epithelial cells[13]. Not unexpected was the finding that the Aedes C6/36 cells were infectable[12], since this was described for other related viruses already[14]. Furthermore, a lot of animal cell lines were described to be susceptible to ZIKV infection[15]. When mice lacking receptors for IFN-α/β (A129) were infected with the ZIKV, viral RNA could be found in the brain, ovary, spleen and liver[16].

In order to further characterize the virus in human cell lines and to identify cell culture systems that allow the robust production of high amounts of infectious viral particles, ten cell lines were comparatively analyzed for their susceptibility to the ZIKV. Keratinocytes (HaCaT) were included in the following experiments, since the skin is the first tissue the virus comes in contact with *via* mosquito bite. Moreover neuronal cells (N29.1 and SH-SY5Y) were of special interest due to the neurological disorders ZIKV infections may cause. Furthermore the infectivity of the well-established standard cell lines 293T cells, CHO cells, Vero cells, A549 cells, HepG2C3A cells, Huh7.5 cells and COS7 cells was studied.

MATERIALS AND METHODS

Cell culture

A549, CHO, COS7, HepG2/C3A, Huh7.5, HaCaT, N29.1, SH-SY5Y, Vero and 293T cells (Table 1) were grown in Dulbecco's modified eagle medium (DMEM) supplemented with 2 mmol/L L-Glutamine, non-essential amino acids, 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified incubator at 37 ℃ with 5% CO_2. Passaging of the cells was carried out three times a week, reaching a maximum density of 90%.

ZIKV strains

The cells were infected with the ZIKV strain French Polynesia (PF13/251013-18) (this clinical low passage strain was kindly provided by Professor Musso, Institute Louis Marlade in Papeete, Tahiti).

Infection procedure

The inoculum for the infection experiments was derived from Vero cells that were infected for 72 h with ZIKV Polynesia. The obtained cell culture supernatant was filtrated and characterized by titration using plaque assays. Defined aliquots were stored at -80 ℃. The cell lines were infected with ZIKV at a MOI = 0.1 for 16 h. The inoculum was removed, cells were washed with prewarmed PBS, cultivated with medium for 32 h and harvested after 48 h if not stated differently.

The obtained cell culture supernatant was filtrated

Table 1 Summary of the cell lines used in this study

Cell line	Species	Tissue	Origin
A549	Homo sapiens; human	Lung epithelial	1972; D. Giard
CHO	Chinese Hamster	Ovarie	1957; T. Puck
COS7	Chlorocebus aethiops African Green Monkey	Kidney	1964; F. Jensen
HepG2/C3A	Homo sapiens Human	Liver	1980; B. Knowels
Huh7.5	Homo sapiens Human	Liver	1980; B. Knowels
HaCaT	Homo sapiens Human	Skin	1988; P. Boulkamp
N29.1	Mus musculus Mouse	Hypothalamus	Cedarlane Laboratories
SH-SY5Y	Homo sapiens Human	Bone marrow Neuroblastoma	1973; J. Biedler
Vero	Chlorocebus aethiops African Green Monkey	Kidney	1962; Y. Yasumura
293T	Homo sapiens Human	Kidney	1973: van der Eb

and characterized by titration using plaque assays. Defined aliquots were stored at -80 ℃. The cell lines were infected with ZIKV at a MOI = 0.1 for 16 h. At the present stage of knowledge no detailed information about the velocity of the infection process in the different cell culture systems is available. To avoid effects that reflect potential differences in the velocity of the infection process cells were infected for 16 h (overnight) to ensure a high infection level. The inoculum was removed, cells were washed with prewarmed PBS, cultivated with medium for 32 h and harvested after 48 h if not stated differently.

Virus titration assay (plaque assay)

Vero cells were seeded at a density of 3×10^5 cells per well in standard six well plates and infected with cleared, serial dilutions of either cell culture supernatant or cleared cellular lysate 6 h later. Another 2 h later, the inoculum was removed and the cells were washed twice with PBS. Then the cellular monolayers were overlaid with DMEM complete containing 0.4% seaplaque agarose. Four days later, the agarose overlay was removed and the wells were washed with PBS. Afterwards, the cells were fixed with 4% formaldehyde for 10 min and stained with 0.1% crystal violet for plaque visualization.

RNA isolation and cDNA synthesis

RNA from total lysate was isolated using peqGOLD TriFast (PEQLAB Biotechnologie GmbH, Germany) according to the manufacturer's protocol. cDNA synthesis was performed after DNA digest with DNaseI (Promega, Mannheim, Germany), using 4 μg total RNA, RevertAid H Minus Reverse Transcriptase and random primer (Thermo Scientific, Dreieich, Germany) as suggested by the manufacturer.

RNA from cell culture supernatant was isolated using QIAamp viral RNA Mini Kit (Qiagen, Hilden Germany) as described by the manufacturer. However, the elution volume was decreased to 40 μL per sample.

Quantitative real-time PCR from total lysate and cell culture supernatant

Quantitative real-time PCR (qPCR) from total RNA was performed as described[17]. All relative quantifications were normalized to the amount of RPL27 transcripts. The following primers were used: Zika fwd (5' agatcccggctgaaacactg 3'-bp 1924-1943), Zika rev (5' ttgcaaggtccatctgtccc 3'-bp 1996-1977), ribosomal protein L27 - RPL27 fwd (5' aaagctgtcatcgtgaagaac 3') and RPL27 rev (5' gctgctactttgcgggggtag 3').

qPCR using RNA isolated from cell culture supernatant was analyzed using Zika LightMix Kit (TIB MOLBIOL, Berlin, Germany) in combination with LightCycler® Multiplex RNA Virus Master (Roche, Mannheim Germany) as described by the companies protocols. In brief, 2.7 μL PCR grade water, 0.25 μL Zika Light Mix, 2 μL Roche Master, 0.05 μL RT Enzyme were mixed with 5 μL purified RNA and measured in the LightCycler 480 or Light cycler 1.2 (Roche, Mannheim Germany) with the following program: (1) RT-Step: 55 ℃/5 min; (2) Denaturation: 95 ℃/5 min; and (3) Cycling (45 times): 95 ℃/5 s, 60 ℃/15 s, 72 ℃/15 s; Cooling: 40 ℃/30 s.

Western blot analysis

The samples were resolved by sodium dodecyl sulfate-polyacrylamid electrophoresis (SDS-PAGE) at 10% and transferred by semi-dry blotting onto a polyvinylidine difluoride membrane (PVDF) (0.45 μm; Carl Roth, Germany). The membrane was blocked with 5% skim milk solution and then incubated with anti NS1 specific antibody at a 1:1000 dilution (Biofront, United States) overnight. Then the membrane was incubated with a mouse specific secondary antibody coupled with horseradish peroxidase at a 1:2000 dilution (HRP) (GE Healthcare, United Kingdom) and signals were detected with X-ray films (GE Healthcare, United Kingdom). Signals were quantified using ImageJ software.

Immunofluorescence analysis

Immunofluorescence staining was analyzed with a confocal laser scanning microscope (CLSM 510 Meta; Carl Zeiss) and ZEN 2009 software. Cells were fixed with absolute ice-cold ethanol for 10 min. ZIKV envelope protein was stained using anti Flavivirus Group antigen Antibody (clone D1-4G2-4-15 from Merck-Millipore, Darmstadt Germany) and a polyclonal rabbit antiserum was used to detect STAT1 (Merck AB16951). As secondary antibodies served Alexa 488 and Alexa 546 (Thermo, Darmstadt Germany). Nuclei were stained with DAPI.

CPE detection

Cytolysis was monitored by LDH release assay (Clontech, Japan) and cell viability was assessed by Presto Blue staining (Thermo, Darmstadt Germany) according to the instructions of the manufacturer. Upon cellular damage lactate dehydrogenase (LDH) is released into the cell culture supernatant. This release is indirectly measured based on a calorimetric assay detecting an enzymatically formed formazan product. Presto Blue is a red compound that is taken up by the cells and due to the reducing interior environment turns into a red color that is detectable at 570 nm.

Transfection and luciferase reporter assay

The used cell lines were transfected with the pISREluc construct (Agilent, United States), using polyethylenimine (PEI) directly after infection. In brief, 3×10^5 cells per six well were infected as described and transfected directly after the addition of virus, using a transfection mix containing 1 μg plasmid DNA, 12 μL PEI (1 mg/mL) in a total volume of 150 μL PBS (1/10 total volume of media). The media was changed the next day and cells were analyzed 48 h post transfection. Here for cells were lysed in a passive lysis buffer (25 mmol/L Tris, 2 mmol/L DTT, 2 mmol/L EGTA, 10% glycerol (v/v), 1% TX-100 (v/v), pH7.5) for 10 min on ice. Afterwards, lysate was cleared by centrifugation at 4 ℃ and 5000 × *g* for 10 min and the luciferase activity of the supernatant was measured in 96 well Orion II plate reader (Berthold) for 10 s after the addition of luciferase buffer (20 mmol/L Tris-HCl pH7.8, 5 mmol/L $MgCl_2$, 0.1 mmol/L EDTA, 33.3 mmol/L DTT, 470 μmol/L Luciferin, 530 μmol/L ATP). Relative light units were normalized to the total protein amount by Bradford protein assay.

Statistical analysis

All statistical analyses were performed with Prism GraphPad 7.0, using multiple *t* tests for determination of *P*-values. Error bars are displayed as value ± SEM.

RESULTS

ZIKV replicates efficiently in various cell lines but not in CHO cells

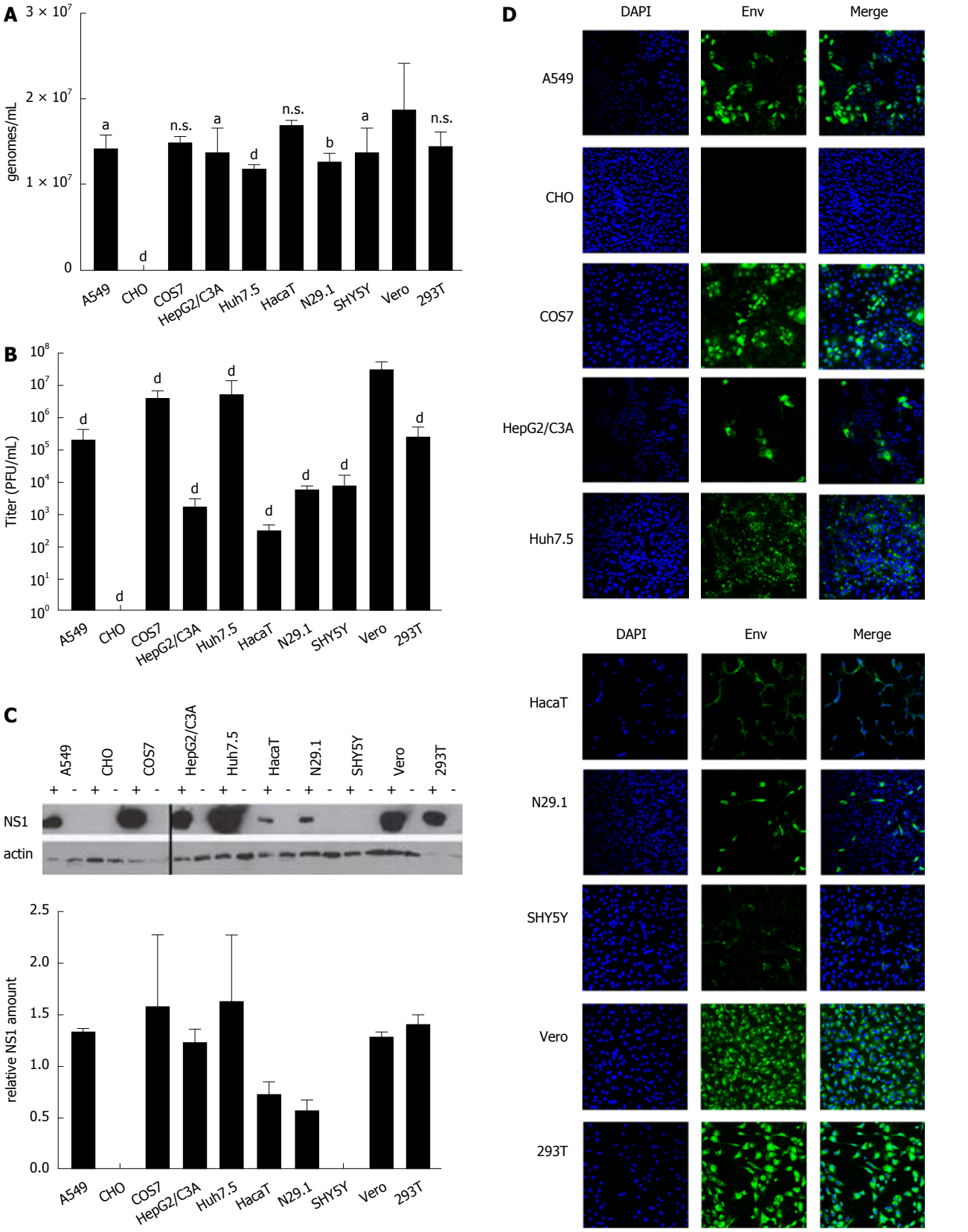
The capacity of various human- and non-human-derived cell lines to produce high amounts of infectious ZIKV particles was analyzed. For this purpose, ten different (human and non-human) cell lines (Table 1) derived from various tissues (neuronal cells, kidney cells, keratinocytes, hepatoma cells and lung epithelia cells) were tested with respect to their susceptibility to ZIKV infection. The investigated cells were infected with an identical MOI of 0.1, using ZIKV Polynesia strain. The intracellular amount of ZIKV-specific genomes was determined by RT-PCR 48 h after infection, revealing that for all analyzed cell lines with the exception of CHO cells a productive infection could be established. With respect to the number of intracellular genomes detected in the various cell lines (Figure 1A), only moderate differences between the permissive cell lines were found. However, the amount of viral genomes must not necessarily correlate with the amount of infectious viral particles. To address this point, the number of infectious particles in the cell lysates was determined. Quantification of the intracellular amount of infectious viral particles by plaque assay (Figure 1B) revealed strong differences between the investigated cell lines of up to 10^5-fold. The highest amount of intracellular infectious viral particles was found for Vero containing 3.6×10^7 PFU/mL followed by the Huh7.5, COS7, 293T and A549 cells. Again, N29.1 and SH-SY5Y cells showed significantly lower amounts of intracellular viral particles (8.7×10^3 - 2.3×10^3 PFU/mL). The HaCaT cells showed besides the CHO cells, which did not contain infectious viral particles, the lowest amount amongst the investigated cell lines (3×10^2 PFU/mL). Comparison between the quantification of the intracellular viral genomes and the infectious viral particles reveals that there is a correlation, but the differences between the various cell lines are much more pronounced with respect to the amount of infectious viral particles in comparison to the viral genomes.

ZIKV-infected cells differ significantly with respect to the intracellular amount of NS1

Quantification of intracellular viral genomes does not automatically reflect replication. To further analyze ZIKV replication, the intracellular amount of NS1 was determined by western blot analysis and referred to the amount of actin (Figure 1C). The quantification of the western blots demonstrates that between the different cell lines significant differences with respect to the intracellular amount of NS1 can be observed. Nearly the same pattern for the amount of NS1 can be observed as found for the intracellular genomes by RT-PCR. A549, COS7, HepG2/C3A, Huh7.5, Vero and 293T cells showed strongest signals, while lower amounts of NS1 were detected in N29.1 cells. No NS1 was measurable in SHY5Y and CHO cells. For the HaCaT cells in contrast to the qPCR data only a low amount of NS1 was observed.

Analysis of the amount and subcellular distribution of ZIKV envelope protein by confocal immunofluorescence microscopy

To estimate the intracellular amount of ZIKV envelope


A
genomes/mL
3 × 10^7
2 × 10^7
1 × 10^7
0
a
d
n.s.
a
d
n.s.
b
a
n.s.
A549
CHO
COS7
HepG2/C3A
Huh7.5
HacaT
N29.1
SHY5Y
Vero
293T
B
Titer (PFU/mL)
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
C
NS1
actin
relative NS1 amount
2.5
2.0
1.5
1.0
0.5
0.0
D
DAPI
Env
Merge
A549
CHO
COS7
HepG2/C3A
Huh7.5
HacaT
N29.1
SHY5Y
Vero
293T

E

Cell line	VTotal cell count	Env pos. cell count	Percentage pos. cells
A549	158	105	66.46
CHO	300	0	0
COS7	493	149	30.22
HepG2/C3A	421	48	11.4
Huh7.5	461	244	52.93
HacaT	263	111	42.21
N29.1	679	54	7.95
SH-SY5Y	487	36	7.39
Vero	371	266	71.70
293T	200	134	67.00

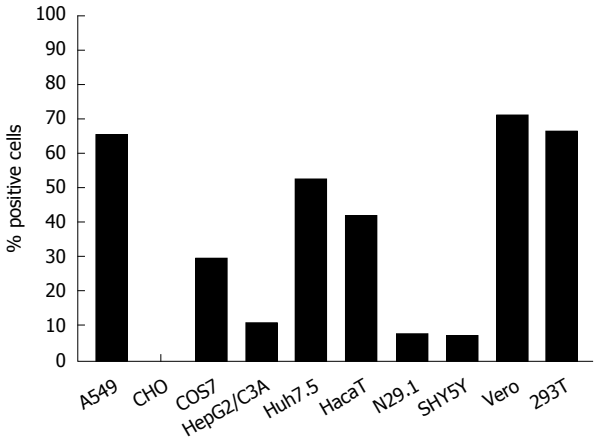


Figure 1 ZIKV-infected cells differ significantly with respect to the intracellular amount of infectious viral particles. A: Cells were infected with an identical MOI of 0.1, using ZIKV Polynesia strain. Forty-eight hours after infection the intracellular amount of ZIKV-specific genomes was determined by RT-PCR. The data are the mean from four independent experiments. Amounts of Zika genomes are calculated using a Zika virus standard. A threshold value of 10 viral genomes was used. The bars represent the standard deviation of the mean. Statistical analysis was done by using 2-way ANOVA with Vero cells as reference value. [a]$P < 0.05$, [b]$P < 0.01$, [d]$P < 0.0001$; B: Cells were infected with an identical MOI of 0.1, using ZIKV Polynesia strain. Forty-eight hours after infection the cells were lysed and intracellular amount of infectious viral particles was determined by plaques assay using Vero cells. The data are the mean from four independent experiments. A threshold value of 10 plaques was used. The bars represent the standard deviation of the mean. Statistical analysis was done by using 2-way ANOVA with Vero cells as reference value. [d]$P < 0.0001$; C: Cells were infected with an identical MOI of 0.1, using ZIKV Polynesia strain. Forty-eight hours after infection the cells were lysed and intracellular amount of NS1 was determined by western blot analysis and referred to the amount of actin. The experiment was done in triplicate; one representative experiment is shown. Two different western blots from two independent experiments were quantified using Image J software. The relative NS1 amount represents the ratio between NS1 and actin; D: Cells were grown on cover slips and infected with an identical MOI of 0.1 using ZIKV Polynesia strain. Forty-eight hours after infection the cells were fixed by ethanol. To quantify the intracellular amount of ZIKV envelope protein and to analyze the subcellular distribution of the envelope protein in the different cell lines, confocal immunofluorescence microscopy was performed using an envelope-specific antibody (green fluorescence). Nuclei were stained by DAPI (blue fluorescence). The pictures were taken at 450-fold magnification; E: In two visual fields the total number of cells were determined by counting the number of DAPI-labelled cells. For quantification of ZIKV-positive cells immunofluorescence microscopy was performed using the envelope protein specific antibody 4G2. The amount of ZIKV-positive cells was determined based on the env-staining. The percentage of ZIKV-positive cells was calculated and depicted in a diagram. ZIKV: Zika virus.

protein and to analyze the amount of infected *vs* non-infected cells, confocal immunofluorescence microscopy was performed (Figure 1D). The staining showed that based on the plaque assays the highest producer cells also showed the best ratio between infected *vs* non-infected cells. The confocal immunofluorescence microscopy shows for the A549, Vero and 293T cells a susceptibility between 72%-66%, while from the Huh7.5 and HaCaT cells approximately 52% and 42% and only approximately 10% of the HepG2/C3A, N29.1 and SH-SY5Y were infected after 48 h. In case of the CHO cells, no specific staining was observed confirming that these cells are not permissive for ZIKV.

Pronounced cytopathogenic effect of ZIKV in A549 and Vero cells

To study the impact of ZIKV on cell viability and integrity in the different analyzed cell lines Presto Blue assays for determination of the cell viability and LDH assays for analysis of the cell integrity were performed. For this purpose the cells were infected for 48 h and stained for Presto Blue assays or the supernatant from ZIKV-infected cells was collected 48 h after infection and the LDH activity was determined (Figure 2). Both assays revealed that ZIKV heavily affects cell integrity/cell viability in A549 and Vero cells. Less cell death was observed for HepG2/C3A, HaCaT and N29.1 cells. Based on the data from these assays and microscopic analysis COS7, 293T and SH-SY5Y cells were found to be most resistant to ZIKV induced cytolysis.

Virus release differs strongly in tested cell lines

To investigate whether the data obtained for the analysis of the intracellular amount of viral genomes and infectious viral particles are reflected by the numbers of genomes and infectious viral particles in the supernatant, media from the infected cell cultures were analyzed 48 h after infection. The RT-PCR (Figure 3A) revealed that in accordance to the results obtained for the quantification of the intracellular genomes, CHO released no viral genomes. As observed for the intracellular amount of viral genomes, there were no major differences in the amount of released viral genomes between the different cell lines. The difference between the highest amount observed for A549, HaCaT and Vero cells on the one side and N29.1 or SH-SY5Y cells on the other side is less than 2 fold. As the amount of viral genomes in the supernatant must not correlate with the amount of infectious viral particles, the number of infectious viral particles in the cell culture supernatants was determined by virus titration assays (Figure 3B). In contrast to the moderate differences that were found analyzing the number of viral genomes, strong differences (more than 10^2-fold) were revealed with respect to the number of infectious viral particles released by the different cell lines. The highest amounts were detected for supernatants derived from Vero-, A549-, COS7-, HepG2/C3A-, Huh7.5-, HaCaT- and 293T-cells that produced

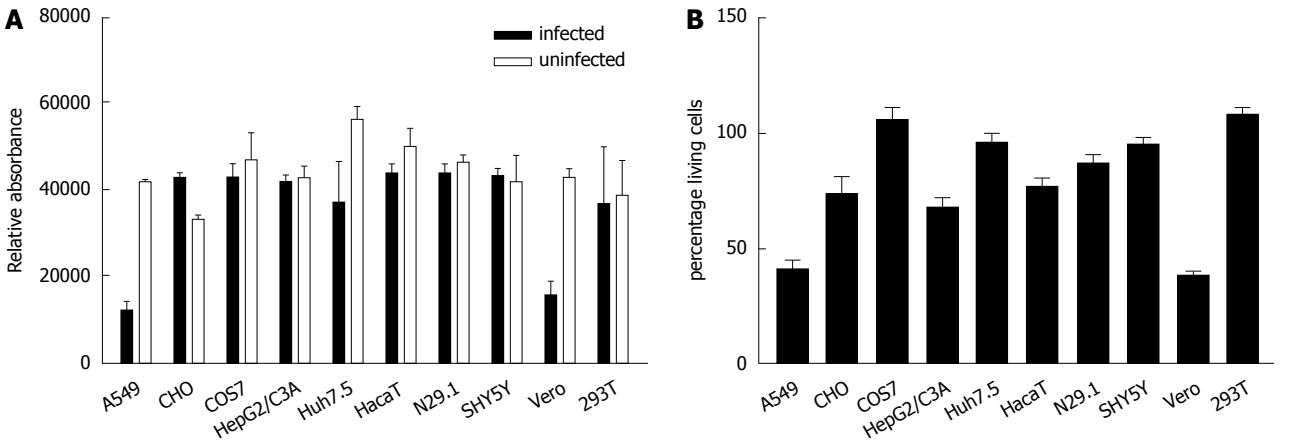


Figure 2 In A549 and Vero cells Zika virus exerts a pronounced cytopathogenic effect. Cells were infected with ZIKV. A: Forty-eight hours cell viability was analyzed by Presto blue assay; B: Cell integrity was analyzed by determination of the LDH-level in the cell culture supernatant. The data are the mean from three independent experiments. The bars represent the standard deviation of the mean.

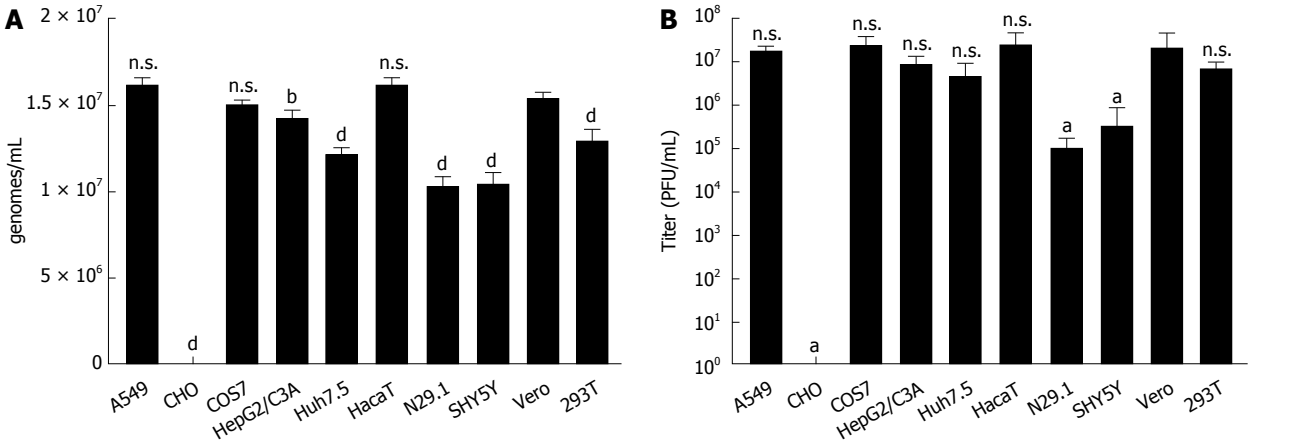


Figure 3 Zika virus-infected cells release comparable amounts of viral genomes but differ significantly with respect to the intracellular amount of infectious viral particles. A: The indicated cell lines were infected with a MOI = 0.1, RNA was isolated from the supernatant 48 h post infection and analyzed by qPCR. Amounts of Zika genomes are calculated using a Zika virus standard. Shown are the amounts of genomes/mL in the supernatant from four independent experiments. A threshold value of 10 viral genomes was used. The bars represent the standard errors of the mean. Statistical analysis was done by using 2-way ANOVA with Vero cells as reference value. $^{b}P < 0.01$, $^{d}P < 0.0001$; B: The indicated cells were infected with a MOI = 0.1 and supernatant was harvested 48 h post infection. To quantify the amount of released infectious viral particles, the obtained supernatants were used for plaque assays on Vero cells. Plaques were visualized and counted 4 d after infection. The data are from four independent experiments. A threshold value of 10 plaques was used. The bars represent the standard errors of the mean. Statistical analysis was done by using 2-way ANOVA with Vero cells as reference value. $^{a}P < 0.05$.

nearly the same quantity of infectious viral particles (about 10^7/mL). The neuronal cell lines N29.1 and SH-SY5Y cells released more than 100 times less infectious viral particles than Vero cells. For CHO cells no significant amount of released viral particles was detectable.

Interferon response does not necessarily correlate with the extent of viral infection

Induction of interferon-stimulated genes (ISGs) was analyzed by luciferase reporter assay using the Interferon-stimulated response element (ISRE) as promoter-driving luciferase expression. Cells were infected as described, transfected with pISREluc plasmid and the cellular luciferase activity was analyzed 48 h post infection (Figure 4A). Luciferase assay showed an induction of ISGs only for the N29.1 cells. In the rest of the tested cells ISGs were slightly repressed. However, staining of STAT1 suggests a delocalization by ZIKV (Figure 4B). If ZIKV was present in the cells it occurred like STAT1 is drawn to the replication factories and no longer is evenly distributed as seen in uninfected cells.

Taken together, these data indicate that the analyzed cell lines strongly differ with respect to the amount of released viral particles, although comparable amounts of viral genomes are detectable in the supernatant. With respect to the identification of cell culture systems that are suitable to produce high amounts of infectious viral particles, Vero- and COS7 cells as a non-human-derived cell lines and Huh7.5-A549 and 293T cells as human-derived cell culture systems were identified.

DISCUSSION

ZIKV first isolated from a sentinel monkey in the Zika

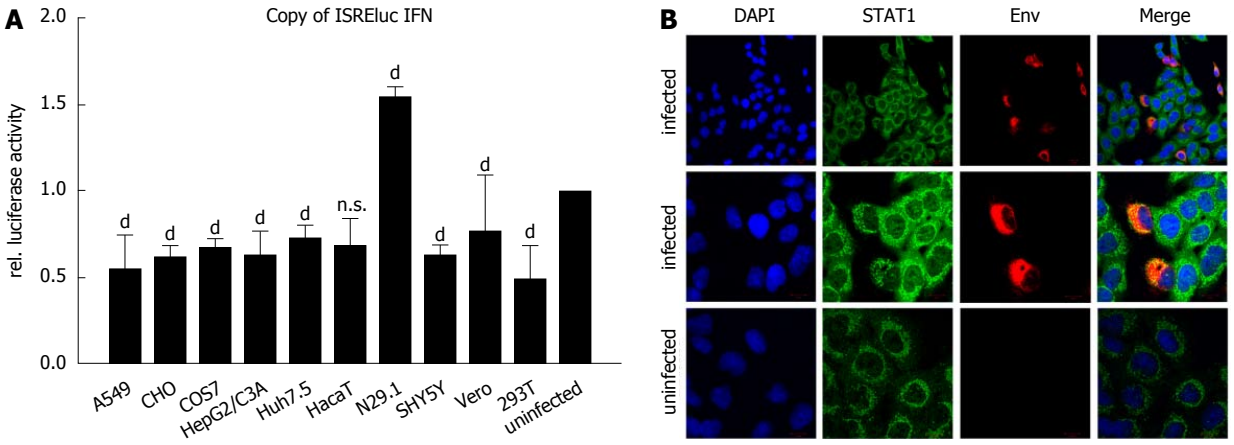


Figure 4 Delocalization of STAT1 in Zika virus-infected cells. A: The indicated cell lines were either infected with a MOI = 0.1 or left uninfected and directly afterwards transfection with the pISREluc reporter was performed. 16 h later the cells were washed once with PBS and luciferase activity was measured 48 hpi. The data are from three independent experiments and for each cell line the uninfected controls were set as one, acting as the reference. The bars represent the standard errors of the mean. Statistical analysis was done by using 2-way ANOVA with Vero cells as reference value. $^{b}P < 0.01$, $^{d}P < 0.0001$; B: Detection of STAT1 by confocal immunofluorescence microscopy of ZIKV-infected and uninfected A549 cells. Cells were grown on cover slips and infected with a MOI of 0.1 using ZIKV Polynesia strain. 6 h after infection the cells were fixed with ethanol. Detection of STAT1 was performed using the green channel and Zika specific staining was performed using an envelope-specific antibody (green fluorescence). Nuclei were stained by DAPI (blue fluorescence). The pictures were taken at 450-fold and 1000-fold magnification respectively (scale bars represent 10 μm). ZIKV: Zika virus.

forest in Uganda in 1947[1] is an emerging virus that has spread over the years from Africa, over Asia, Micronesia, French Polynesia to Brazil[18]. Since the Brazil epidemic in 2015/2016[19] ZIKV research has increased dramatically. In this study a ZIKV isolate from Polynesia that also belongs to the Asian lineage like the ZIKV strain causative for the epidemic in Brazil, was used to characterize its potential to infect various human cell lines with the aim to provide cell culture models for investigating the ZIKV life cycle in more detail and to test the suitability of various cell culture systems to produce high amounts of this virus[20]. The French Polynesia strain was utilized since this was described first to cause GBS and microcephaly, like later for the Brazil outbreak[4,7,20]. The virus targets different cell types such as macrophages, fibroblasts, trophoblasts as well as mesenchymal stem cells[21].

In light of the correlation between ZIKV infection of pregnant women and the development of microcephaly in the fetus it is of major interest that ZIKV can be detected in the maternal decidua, fetal placenta and umbilical cells.

In interferon receptor type I-deficient mice (IFNAR KO) ZIKV causes systemic infections in all tissues providing a useful tool for drug target testing[16,22-24]. Moreover, several publications have shown to replicate ZIKV in various primary cells like neuronal cells, dendritic cells or keratinocytes[12,14,25,26]. Immortalized cells are already described for infection with ZIK[11,12,14,27].

In this comparative study here, ten cells lines were tested for their capacity to support replication and production of infectious virus. After infection of the cells, the ongoing replication was monitored by qPCR, western blot and immunofluorescence microscopy analysis, while the capacity to produce infectious virus was investigated by qPCR and plaque assay. It was found that ZIKV replicates in neuronal cells, keratinocytes, lung carcinoma cells, liver carcinoma cells and kidney cells. Vero cells always served as positive control and standard for quantification. ZIKV showed best replication and virus production in A549, COS7, Huh7.5 and 293T cells, followed by HepG2/C3A and HaCaT cells. Significantly lower support was measured for N29.1 and SH-SY5Y cells. In contrast to these cells, CHO cells could not be infected. The finding that 293T cells are susceptible to ZIKV stands in contrast to a previous publication by Hamel *et al*[12] (2015). Huh7 cells have also been used in some studies and demonstrated to support viral replication[11], but here we used the Huh7-derived Huh7.5 cells clone[28] that has a defect in the RIG-I gene[29]. However, comparable titers were reached for the Huh7 cells measured by Cortese *et al*[11] and the Huh7.5 cells in our hands, so that there seems to be no benefit using the Huh7.5 cells at least for the production of high titer viral stock, indicating that ZIKV replication is not significantly affected by the functionality of the RIG-I gene. ZIKV propagation in primary keratinocytes has been shown already[12]. Here we used the immortalized keratinocyte cell line HaCaT[30] which turned out to be susceptible to viral infection and also produced a good viral titer. Moreover, the virus replicates very efficiently in COS7 cells[31], showing release of infectious virus to a titer of over 10E7 viral particles/mL. This was not unexpected since these cells, like Vero cells, derive from the kidney of an African green monkey. By using a MOI of 0.1, which is in the lower range if compared to Dengue virus titers used for infections in cell culture, we assured to only identify cells that support viral infection efficiently. The measured viral genomes in the corresponding supernatants resembled the findings from the intracellular quantification, in which the measured genomes in case of CHO cells represent

input RNA from the infection procedure. However, comparable amounts of viral RNA were observed inside and outside of the cells.

When comparing the intracellular and the extracellular titers of infectious virus, in general higher titers were measurable in the supernatant of the cells compared to the intracellular amount of virus. But for the high producer cell lines like A549, COS7, Huh7.5, Vero and 293T nearly the titers measured outside the cells were reached already inside the cells. This is also reflected by the detection of NS1 in cellular lysate by western blot and by analyzing the Env-protein by immunofluorescence. Here also more NS1 was detectable for the high producers and more infected cells were visualized. However, for unknown reasons only low titers were detected inside the HepG2/C3A cells, but nearly 10E4 more infectious virus was secreted by these cells. This enhancement of viral release may be based on efficient packaging and export of the viral cargo or a lower turnover of viral proteins.

To investigate if the activation of IFN-signaling is causative for the diverging susceptibility of the analyzed cell lines, the induction of ISGs was monitored by pISRE-luciferase reporter experiments upon viral infection. The luciferase assay showed for all infected cell lines a reduction on ISRE activity, except for N29.1 cells. Inhibition of Type Ⅰ and Ⅲ IFN production by interfering with the STAT signaling has been demonstrated in several recent publications for the ZIKV[32–34]. Particularly NS5 is described to counteract IFN signaling by binding STAT2 and to promote STAT2 degradation by the proteasome[32]. Moreover, NS1, NS4B and NS2B3 were also shown to inhibit IFN signaling[34]. In contrast to wt mice having a functional adaptive immune response, IFNAR KO mice are susceptible to ZIKV infection[16,23]. This also emphasizes the importance of the IFN response which has to be trapped by the virus in order to establish infection. Astonishingly, the ISRE promoter element was also reduced in its activation in CHO cells, although they do not support viral infection and replication. Since it is not known if the lack of susceptibility of CHO cells for ZIKV infection is due to impaired attachment, entry or post entry steps there exist a variety of possibilities that could lead to an interference with the interferon signaling. The results from the luciferase assay were strengthened by STAT1 staining in infected A549 cells. The changed distribution in infected *vs* uninfected cells is obvious, but from these experiments it stays uncertain if this is causative for the reduced ISRE activation. The results of this study support ZIKV research by providing different cell culture models based on various tissues, so that the information at hand enables the investigation of ZIKV life cycle in more detail. Also drug testing and pathogenicity studies can be fostered by the shown cell culture models susceptible to ZIKV.

ARTICLE HIGHLIGHTS

Research background

Zika virus (ZIKV) is an emerging virus transmitted mainly by mosquitos, that has spread during the last decades from Africa, to Asia, over Micronesia to the Americans causing an epidemic in Brazil in the years 2016/2017. In order to propagate the virus in cell culture we investigated various cell lines for their susceptibility to ZIKV infection.

Research motivation

To date ZIKV is mainly propagated in Vero cells derived from kidney epithelial cells from African green monkey. This study aimed to investigate the potential of various cell lines to support the viral life cycle in order to provide researchers with suitable cell culture systems for different issues in the field of ZIKV research.

Research objectives

The objectives of this research were to investigate ten human and non-human cell lines from various tissues (*e.g.*, hepatocytes, keratinocytes and neuronal cells) with regard to their intracellular amount of viral genomes and infectious viral particles upon ZIKV-infection. Moreover, the amount of secreted viral genomes and infectious viral particles was analyzed in the cell culture supernatants. Furthermore, the amount of infected cells was analyzed by immunofluorescence using an Envelope-specific antibody and the amount of NS1 was analyzed by western blot. In order to draw a conclusion whether parts of the innate immune response are responsible for the found differences in viral support, STAT1 distribution and expression was analyzed.

Research methods

Quantification of viral genomes was performed by qPCR. For the detection of genomes from whole cell lysate a standard PCR protocol with SYBR green was used with cDNA as template transcribed from total RNA that was isolated with a Tri-reagent. Viral genomes released into the cell culture supernatant were isolated with a viral RNA isolation kit and subjected to a Taqman-PCR based on a ZIKV-Lightmix Kit. The detection of infectious virus was performed by virus titration assay using serial dilutions from the supernatant or from cleared cellular lysates. The amount of infected cells was analyzed with immunofluorescence microscopy by using an Env-specific antibody and with western blot using NS1-specific antibody. The effect on the innate immunity was monitored by luciferase-reporter assay and STAT1 analysis distribution in the immunofluorescence microscopy.

Research results

All investigated cell lines except CHO cells supported infection, replication and release of ZIKV. While in infected A549 and Vero cells a pronounced cytopathic effect was observed COS7, 293T and Huh7.5 cells were most resistant. Although the analyzed cell lines released comparable amounts of viral genomes to the supernatant significant differences were found for the number of infectious viral particles. The neuronal cell lines N29.1 and SH-SY5Y released 100 times less infectious viral particles than Vero-, A549- or 293T-cells. However there is no strict correlation between the amount of produced viral particles and the induction of an interferon response in the analyzed cell lines.

Research conclusions

The results presented so far provide a toolbox of cell culture systems for ZIKV research in general. However, the analyzed cells differ strongly with respect to the amount of released viral particles, whereas the amount of genomes amongst the cells in the supernatant and inside of infected cells are more or less equal. This is an important finding, since a lot of research and diagnostic is based on qPCR analysis only.

Research perspectives

Further research should aim on the differences of released viral genomes *vs* released infectious virus. Are there differences in the release pathway? Which pathways are used for viral egress? Why are certain cell lines not succeptible?

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