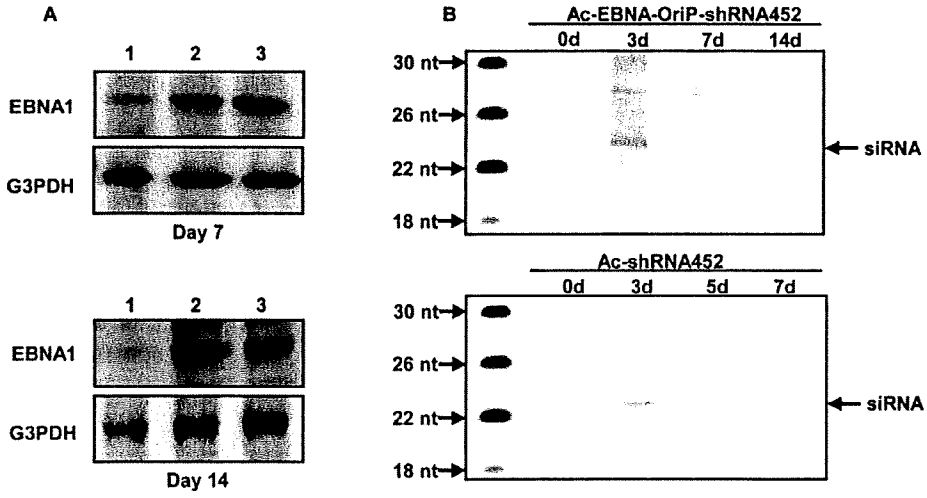

Figure 4
Detection of EBNA1 protein and siRNA in Ac-EP-shRNA452 infected cells. A Western blot analysis of EBNA1 expression in baculovirus-infected Huh7 cells. Cell lysates were prepared 7 days and 14 days post-infection from cells infected with different viruses. Lane 1: Ac-sh452; lane 2: Ac-EP-sh452; lane 3: Ac-EP-control-shRNA. **B** Expression of siRNA by a baculovirus vector. To demonstrate the intracellular expression of the shRNA construct in the respective siRNA, Huh-7 cells were infected with Ac-EP-shRNA452. The mixture was run on a 15% polyacrylamide TBE urea gel after 3, 7, and 14 days.

gated anti-goat antibody (Sigma Chemical Co., St. Louis, MO) was used as the secondary antibody.

RNA purification and real-time RT-PCR

Total RNA was isolated from the cells using a mirVana miRNA Isolation Kit (Ambion, Austin, TX). Real-time RT-PCR was performed using the following primers located in the HCV core region: forward primer (813-833 nt), 5'-CTGGAGGACGGCGTGAATTAT-3'; reverse primer (938-957 nt), 5'-CGTTCGTGACATGGTATATC-3'. HCV-specific RNA was detected by real-time PCR as an increase in SYBR Green I fluorescence on an ABI PRISM 7700 (Applied Biosystems, Foster City, CA). The 18S rRNA housekeeping gene was used as a control for normalization. Each real-time PCR assay was performed in triplicate.

Cytotoxicity assay

NNC#2 cells (2×10^4 cells/mL) were seeded into 96-well microtiter plates and incubated in the presence of various concentrations of the test compounds. The dilutions ranged from 1 to 5-fold, and 9 concentrations were examined. All of the experiments were performed in triplicate. After 3 days culture at 37°C in a CO_2 incubator, cell viability was quantified using a colorimetric BrdU Cell Proliferation enzyme-linked immunosorbent assay according to the manufacturer's instructions (Roche Diagnostics GmbH). The absorbances were read by a microcomputer-controlled photometer (Titertec MultiscanR; Labsystem Oy, Helsinki, Finland) at 405 nm. These values were then translated into percentages per well.

Baculovirus transfer vector constructs

We designed baculovirus transfer vectors expressing shRNAs against the following region of the HCV core-protein sequence: nucleotides 452-472, which contains the nuclear localization signal site (pU6-core-shRNA452) [31]. The following site in the core region of the common sequence of the HCV strain M1LE (GenBank accession number AB080299) was chosen as the target for the shRNA: 5'-GCCGCGCAGGGGCCCCAGGUU-3' (shRNA452). Sense and antisense strands of shRNA oligonucleotides were synthesized, annealed at 95°C for 3 min, and then slowly cooled in phosphate-buffered saline (pH 7.4, containing 50 mM NaCl). The oligonucleotides contained the loop CCACACC sequence, and KpnI and BamHI ends, which were inserted into a pU6 vector, based on pSV2-neo. A Pol III-type U6 promoter allowed for constant expression of the shRNAs. Fragments of U6-core-sh452, ranging from the *Eco*RI site upstream of the U6 promoter to the BamHI site downstream of the terminating sequence, were sequenced and then inserted into the cloning site of the baculovirus transfer vectors pVL1392 and pVL1393 (BD Biosciences, San Jose, CA) in an opposite orientation to the polyhedrin promoter to create pVL1392-core-shRNA452 and pVL1393-core-shRNA452. A spacer was inserted between the inverted sequences to form a hairpin structure, and to enhance its stability.

The EBV *EBNA1* and *OriP* gene sequences were obtained from the pCEP4 plasmid (Invitrogen). The *EBNA1/OriP* sequence was digested with restriction enzymes *Eco*RI and

SalI, and inserted into the *EcoRI* and *XhoI* sites of pVAX1 (Invitrogen). The cytomegalovirus (CMV) promoter was amplified by PCR using pCEP4 as the template. The CMV promoter was inserted into the *Hind*III and *EcoR*I sites upstream of the *EBNA1/OriP* sequence. The CMV-EBNA1/OriP unit was digested with *PmeI*, and inserted into the *NaeI* site of the baculovirus transfer plasmid pVL1392 (BD Biosciences) to construct pVL1392-EPCMV. Fragments of U6-core-sh452, ranging from the *NotI* site upstream of the U6 promoter to the BamHI site downstream of the terminating sequences, were sequenced and then inserted into the cloning site of the pVL1392-EPCMV baculovirus transfer vector to produce the plasmid pVL1392-EP-shRNA. Scrambled shRNA (control-shRNA) cloned into the same vector was used as a negative control (pVL1392-EP-control-shRNA) in all experiments.

Preparation of baculoviruses

Recombinant baculovirus containing the shRNA genome (Ac-shRNA) was generated by homologous recombination of the transfer vector and linearized baculovirus DNAs (BD Biosciences) following previously published procedures [39].

Measurement of HCV core protein

AcU6-HCV-core-shRNAs or Ac-EBNAU6-core-shRNAs were used to infect HCV replicon cells. After 3 days, intracellular HCV core-protein levels were measured using a fully automated HCV core-protein antigen chemiluminescent enzyme immunoassay (CLEIA) according to the manufacturer's instructions [40,41]. The relative chemiluminescence unit was measured and used to determine the concentration of the HCV core antigen according to a standard curve generated using recombinant HCV core antigen. The concentration was expressed in units of femtomole/L (fmol/L). Each CLEIA assay was performed in triplicate.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

HS designed the study, performed all of the experiments, and drafted the manuscript. NM participated in the design of the EBNA1/OriP-baculovirus transfer vector construct experiments. TS and MOOC, participated in the design of recombinant baculovirus experiments. HT conceived the study, participated in its design and co-ordination, and helped to draft the manuscript. All authors have read and approved the final manuscript.

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Original Article

HIV/AIDS Acquisition and Transmission in Bangladesh: Turning to the Concentrated Epidemic

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SUMMARY: A seventh round behavioral and serological surveillance found that the HIV epidemic had remarkably increased to 7% among intravenous drug users (IDU) in Central Bangladesh, indicating the urgent need to increase prevention. The main purposes of this study were to find out, by collecting data and the necessary information from sero-surveillances, published reports, and articles, what the prevalence of HIV/AIDS is, and what the acquisition and transmission routes are. In addition, trends in HIV-related risk behaviors among recognized high risk groups were observed, and estimations and projections of HIV transmission up to the year 2020 presented. The Estimation and Projection Package was used to estimate and project HIV transmission. The study results reveal that Bangladesh is a low prevalence country which is turning into one with a concentrated epidemic due to the high HIV prevalence rate of IDU (7%) among the most-at-risk groups. Within this at-risk population, IDU have the highest prevalence rate of HIV transmission, followed by female sex workers, clients of sex workers, and men who have sex with men. If the transmission rate continues to increase, the situation will be uncontrolled. Therefore, there is an urgent need for a comprehensive prevention program to control the spread of HIV.

INTRODUCTION

In South Asia, the HIV epidemic is quite heterogeneous in its dynamics and scope. Bangladesh borders India and Myanmar and is in close proximity to Nepal, countries where the epidemic is severe. India alone has more than half (2.5 million) of all the people living with HIV/AIDS in Asia, with a prevalence rate of 0.36% (1). Bangladesh is considered to be at risk for a large-scale HIV epidemic because of the variety and gravity of risk factors which cause the spread of HIV. In Bangladesh, the first HIV case was detected in 1989, and since then the cases have been steadily increasing, as have all the potential risk factors. The HIV prevalence in the general population appears low (<0.2%), and is estimated as <1% in all risk groups except for injecting drug users (IDU) (7%) (2). However, modeling studies show that an uncontrolled HIV epidemic among drug injectors can accelerate the sexual epidemic and lead to a far greater number of sexually transmitted infections (STI) (3). Although the exact number of HIV cases is not known, by December 1, 2007, 1,207 cases of HIV had been confirmed; of these people, 365 had developed AIDS and 123 had died, a much higher number than in previous years (2). In Bangladesh, surveillance for HIV infection is conducted annually among the population groups most vulnerable to HIV infection. Since 1998, the Government of Bangladesh has been conducting surveillance (known as 2nd generation surveillance) for HIV (1-5) which includes serological and behavioral surveillance; 2nd generation surveillance attempts to capture the potential diversity of HIV distribution by classifying an epidemic into low, concentrated, and generalized categories, and sampling population groups based on the epidemic situation in the country. On behalf of the Government of Bangladesh, the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) has conducted the serological component for each of the rounds, while other organizations have conducted behavioral surveillance. Surveillance is conducted among population groups that are most vulnerable to HIV infection. They include male and female sex workers, transgender persons (hijra), IDU, men who have sex with men (MSM), clients of sex workers such as "babus", who are regular partners of female sex workers in brothels, patients with symptoms of STI, and transport workers, including truckers, rickshaw pullers, launch workers, and dockworkers. Among the types of surveillances conducted, 7th round sero-surveillance provides an exact picture of the rising prevalence of HIV, the high prevalence of active syphilis, and the high prevalence of risky sexual and injecting behaviors among the recognized high risk groups (Tables 1-3). These indicate both the likelihood of incomplete reporting and the potential for a rapid growth in the epidemic. In the absence of a comprehensive case reporting system, Bangladesh has more HIV cases than officially reported. Limited facilities for sentinel surveillance and voluntary counseling and testing, as well as the social stigma and discrimination attached to HIV, contribute to an understatement of the real incidence of HIV. In addition to the risks posed by sexual and other behaviors among particular groups of people, a range of structural factors heighten the vulnerability of Bangladesh's general population to an HIV epidemic. In reality, Bangladesh is near the bottom of most league tables ranking global development indicators, which include widespread poverty and inequality, a high level of adult illiteracy, the low social status of women, the trafficking of women into the commer-

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Table 1. Knowledge and behavior indicators for Bangladesh, 2005 and 2007

Indicator	Population group	Indicator value (%)	
		2005	2007
% of young women and men aged 15-24 who both correctly identify ways of preventing the sexual transmission of HIV and who reject major misconceptions about HIV transmission	Males	NA	24.1
	Females	NA	20.6
	All (15-24) years	NA	22.3
% of most-at-risk group of population who both correctly identify ways of preventing the sexual transmission of HIV and who reject major misconceptions about HIV transmission	FSW	24.0	30.8
	Male CSW	28.2	29.6
	MSM	13.2	27.3
	IDU	14.3	20.2
	All risk groups	17.0	25.9
% of young women and men aged 15-24 who have had sexual intercourse before the age of 15	Males	NA	4.0
	Females	NA	0.8
	All (15-24) years	NA	2.3
% of women and men aged 15-49 who have had sexual intercourse with more than one partner in the last 12 months	Females	NA	NA
	Males	NA	17.5
% of women and men aged 15-49 who had more than one sexual partner in the past 12 months reporting the use of a condom during their last sexual intercourse	Females	NA	NA
	Males	NA	35.2
% of female and male sex workers reporting the use of a condom with their most recent new client	FSW	30.9	66.7
	Male CSW	44.1	43.7
% of men reporting the use of a condom the last time they had anal sex with a male partner	MSM		
	Commercial sex	49.2	29.5
	Non-commercial sex	37.0	24.3
% of injecting drug users reporting the use of a condom the last time they had sexual intercourse	Male IDU		
	Commercial sex	23.6	44.3
	Non-commercial sex	18.9	30.5
	Female IDU		
	Commercial sex	78.9	54.8
	Non-commercial sex	43.9	42.1
% of IDU reporting the use of sterile injecting equipment the last time they injected	Male IDU	51.8	33.6
	Female IDU	60.0	73.8

Source from reference (37).
FSW, female sex workers; CSW, commercial sex workers; MSM, men who have sex with men; IDU, injecting drug users; NA, not available.

Table 2. Impact indicators for Bangladesh, 2005 and 2007

Indicator	Population group	Indicator value (%)	
		2005	2007
% of most-at-risk population who are HIV infected	Female CSW	0.3	0.2
	Male CSW	0.0	0.7
	MSM	0.4	0.2
	Male IDU	4.9	7.0
	Female IDU	–	0.8
	All risk groups	0.6	0.9

Source from references (2) and (20).
Abbreviations are in Table 1.

Table 3. Infection levels among most-at-risk groups for the populations

Surveillance round	Year	No. tested	HIV (%)
1	1998-1999	3,886	< 1% (0.4)
2	1999-2000	4,634	< 1% (0.2)
3	2000-2001	7,063	< 1% (0.2)
4	2002	7,877	< 1% (0.3)
5	2004	10,445	< 1% (0.3)
6	2005	11,029	< 1% (0.6)
7	2006	10,368	< 1% (0.9)

Source from reference (11).

cial sex industry, high infant and maternal mortality, high population mobility, including rural-urban, interstate, and international labor migration, and cultural impediments against discussing or addressing sexual issues.

Biological markers for STIs such as hepatitis C, which is transmitted through blood, are very high in some of the key vulnerable groups, including commercial sex workers (CSW) and IDU. Women who inject are often involved in commercial sex, which heightens their risk of acquiring HIV, and they are thus more likely than their male counterparts to be infected with sexually transmitted diseases (STD). STIs are low in the general population, but are higher in the bridging population groups, such as truckers, and higher yet in CSW. Mobile population groups such as truckers and migrant workers are at high risk for contracting HIV infection (4). Chan and Khan (5) suggested a likely association between HIV risk factors in IDU and other groups, such as MSM. Passive case reporting suggests that another population group is more vulnerable to HIV may be migrant returning from jobs overseas or through cross-border traffic to regions of high prevalence (Table 4). In this regard, Gazi et al. (6) has identified boatmen as a bridging population of HIV/AIDS between the high-prevalence country of Myanmar and the low prevalence country of Bangladesh. All these potential factors fuel the rise in HIV acquisition and transmission. Consequently, the HIV transmission rates among the risk groups as well as the general population is increasing steadily, even while it is remarkably decreasing in some Asian countries (1). If this situation continues, Bangladesh will face a crisis that, as a developing country, it does not have the resources to tackle; it will be unable to mitigate the harmful impact of widespread HIV/AIDS, and will especially be unable to afford the medication costs. Even if only 1% of the general population be-

Table 4. Size estimates of most-at-risk groups in Bangladesh and average estimated number of population living with HIV/AIDS (PLHA) in each group, 2004

Most-at-risk group	Size estimate low	Size estimate high	Average estimate of PLHA
Injection drug users	20,000	40,000	444
Male CSW and MSM	40,000	150,000	450
Brothel-based CSW	3,600	4,000	55
Street-based CSW	37,000	66,000	453
Hotel-based CSW	14,000	20,000	128
Clients of female CSW	1,882,080	31,368,000	1,882
Transgender	10,000	15,000	62
Returnee external migrant	268,000	536,000	3,015
National total most risk groups	2,274,680	39,678,000	6,489
National total population at lower risk[1)]	1,191,559	2,012,375	1,188
Estimated national total average number of the PLHA		7,677	
National range PLHA: ~700 - 19,000		National average PLHA: ~8,000	

Source from reference (8).
[1)]: Partners of members of most-at-risk group for populations, tuberculous patients, and blood transfusion recipients.

comes infected with HIV, there will be 1.5 million people infected. Therefore, there is a pressing need to take steps to prevent this from occurring. The specific objectives of this study were to give a comprehensive, up-to-date overiew of HIV/AIDS in Bangladesh by finfing out what the current acquisition and transmission routes are as well as to examine the trends in HIV related risk behaviors among recognized high risk groups. Estimations and projections of HIV transmission up to the year 2020 are provided, and recommendations presented for future interventions. It is the authors' hope that this study will be helpful to government policy makers and non-government organizations (NGO) as well as to researchers.

MATERIALS AND METHODS

The data was collected from behavioral and serological surveillances and reports published by UNAIDS, WHO, ICDDR,B, and in journals. The updated UNAIDS/WHO Estimation and Projection Package (EPP, 2007) (7) was used to provide the future predictions of HIV transmission in Bangladesh up to the year 2020. The software EPP is generally used to estimate and project the national HIV/AIDS epidemic in a particular country. National HIV epidemics are usually composed of multiple epidemics in different populations and different geographic areas. To reflect this, one of the fundamental principles underlying EPP is that epidemic curves can be developed separately for different populations and then combined to produce a single epidemic curve which estimates HIV prevalence at a national level. They do contain HIV trend data and population characteristics, along with a mathematical curve that fits through those HIV trends. Also, the estimates produced by EPP can be exported to Spectrum (another UNAIDS/WHO Epidemic Software), and used to develop further estimates of the impact of the HIV epidemic. EPP uses available surveillance data to estimate the trends over time of the adult prevalence of HIV at the national level for either concentrated or generalized epidemics. In this study, EPP was used to estimate the trend for a concentrated epidemic. Because of the rapid spread of HIV in one defined subpopulation (IDU), but is not well-established in the general population, most often more than one subpopulation (CSW, clients of CSW, and MSM) with higher risk and HIV prevalence is consistently over 5% in IDU. EPP estimates the trends over time of HIV prevalence by fitting an epidemiological model to the surveillance data provided by HIV sentinel surveillance systems. Modeling and projections has been determined that a model with four parameters is well suited to fitting HIV epidemic curves. Briefly, the four parameters are:

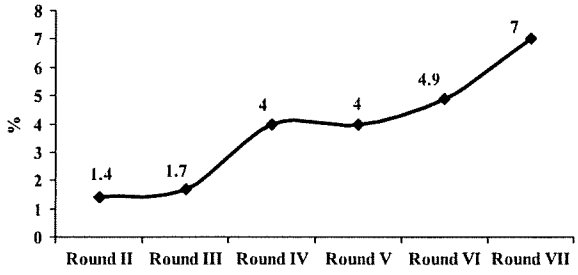
t_0–the start year of the HIV/AIDS epidemic, r–the force of infection, f_0–the initial fraction of the adult population at risk of infection, and Φ–the behavior adjustment parameter. A large value of r will cause the prevalence to rapidly increase, while a small value will cause it to increase slowly. The parameter f_0 determines the peak level of the epidemic curve. The parameter Φ determines how the proportion of new entrants in the adult population who are at risk of HIV infection changes over time. If Φ is negative, people reduce their risk in response to the epidemic and the curve shows a sharper prevalence decline after the peak. If Φ is zero, the proportion at risk remains constant, and the prevalence declines after the peak as people die. If Φ is positive, the risk actually increases over time, and the prevalence falls less quickly or stabilizes at a high level.

Risk group populations and their sexual behaviors: Youth (15 - 24 years) comprise almost one-sixth of the total population of Bangladesh, and are at particular risk of contracting HIV and STIs because of their limited access to sexual and reproductive health information and services. Youth are infected and transmit HIV to the general population through sexual contact and sharing needles. A nationally representative survey of youth was conducted in 2005 by ICDDR,B; Australasian Centre for Policing Research (ACPR) and the Population Council in order to better understand the extent of young peoples' knowledge about HIV and their use of condoms (8). The study results found a generally low level of knowledge about HIV transmission and prevention (22.3%) among both young males and females. In addition, about 22% of unmarried males reported having premarital sex, with one in four reporting visiting a CSW, and half reporting that they did not use condoms.

There are a number of factors that place susceptive groups in Bangladesh at high risk for a rapid increase in HIV infection, an increase that would, by extension, lead to an expanded epidemic of HIV in the general population and other at-risk groups. Within these groups, there is a disproportionate burden of diseases, including STDs. While the underlying social

determinants of health such as poverty, poor access to medical care, low-level of nutritional status, illiteracy, and a poor health care structure are the most likely root causes of these diseases, the more proximal causes include low condom use, a high number of sexual partners, and relatively low HIV-related knowledge and awareness. Only the testing for HIV in donated blood can indicate the HIV prevalence in the general population. In reality, the population at risk in Bangladesh is potentially huge (Table 4). The 2004 estimation of HIV prevalence among risk groups was 0.3%, but in 2007, it was three times greater (0.9%) (Table 3). Almost all sero-surveillance surveys have focused on known at-risk groups such as IDU, CSW, clients of CSW, MSM, heroin smokers, and mobile populations, although several of these populations have posed the most threat to the entire population. The National Surveillance for HIV in Bangladesh also found that a large proportion of transport workers reported having both commercial and non-commercial sex partners, and that condom use was very low (2). In this study, the most-at-risk groups for the populations were identified as IDU, CSW, clients of CSW, and MSM. These four types of populations have very high HIV transmission rates, and the prevalence of HIV among these populations is the primary reason why Bangladesh is so vulnerable (Tables 2-4 and Figure 2).

IDU: IDU are driving the HIV epidemics in many countries, and account for almost a third of new infections outside Sub-Saharan Africa (1). Across the estimated 13 million IDU globally, there is great variation in drug use patterns, behaviors and contexts. The National Assessment of the Situation and Response to Opioid/Opiate Drug Use in Bangladesh (NASROB) first documented the changing patterns in drug use and the introduction of heroin in the mid-1980 (9). By the 1990s, injecting drug use had become more common in Dhaka and Rajshahi (10), and NASROB found the majority of districts surveyed had IDU and heroin smokers. The risk of an impending concentrated HIV epidemic among IDU has been documented in a city in central Bangladesh, where HIV prevalence rose dramatically from 1.7 to 7% in 6 years (Figure 1). Sharing injection paraphernalia is common; 86% in Central A and 63% in Southeast D borrowed needles in the past week in 2003-2004 (11). This was evidently caused by the common practice of using contaminated injecting equipment; 67% of injectors reported using unsafe injection practices (2). The epidemic in IDU is largely confined (10%) to one neighborhood in Dhaka, which can be considered to be the epicenter. There are significant potential factors for sexual spread to the remainder of the population, as IDU engage in more risky sexual behaviors and crimes than non-IDU. IDU are also mobile, traveling from one city to another and sharing injection equipment in different cities. Thus, mobility is another major factor that increases the risk of acquiring and spreading HIV infection. Clearly, IDU networks overlap with other large at-risk populations, such as CSW and their clients. Of particular concern is the fact that there are about 50,000 drug addicts in the country, and many of them are beginning to share injection syringes and needles. Figure 2 clearly explains the HIV transmission and acquisition routes among the risk groups, as well as why HIV infection is transmitted so easily among IDU. The accuracy of the 2004 size was estimated of 25,000-40,000 IDU, which is 0.05% of the country's adult population (Table 4). The 7th round sero-surveillance found that 63% of female IDU (n = 135) were current CSW and 9.9% had active syphilis. Of those, 0.8% were HIV positive. More than 80% of the male drug users reported sex with multiple partners (12). An HIV prevention program in Dhaka found that after one year, 78% of a cohort of 3,200 IDU continued stably exchanging needles and syringes, but their rate of reported condom use in commercial sex encounters remained disturbingly low (13). Group hire sex was also common among IDU, with up to one in six (8.0-17.7%) reporting having engaged in group sex (14). Group sex may be encouraged by financial constraints, with male clients pooling



Source from reference (2).

Fig. 1. HIV Prevalence among injecting drug users, Bangladesh, 1999-2006.

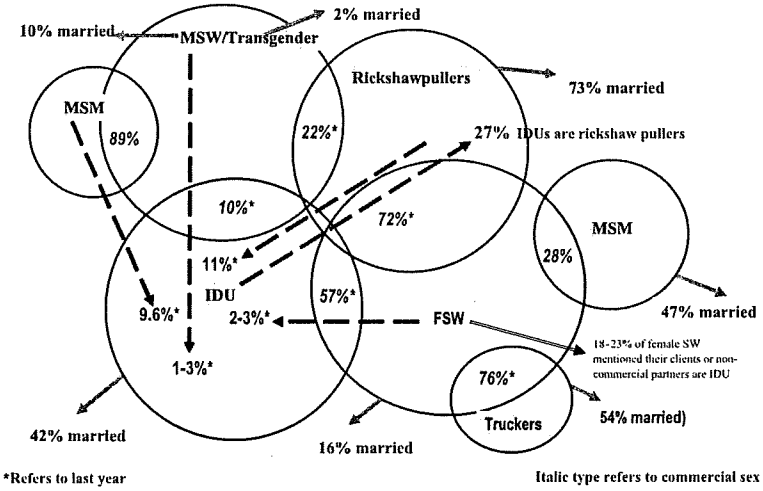


Fig. 2. Potential spread of HIV from most risk groups for the general population in Dhaka City, Bangladesh according to the 4th round sero-surveillance. Abbreviations are in Table 1.

money to share a female CSW (15). It is a particularly risky activity, for men take the additional risk of being exposed to the semen of other men, and the woman is likely to experience trauma and abrasion (16). Among 505 'drug addicts' studied in Dhaka, those who were HIV positive (3.7%) were mainly IDU, and every IDU with HIV reported sharing needles. Those drug users with HIV were also more likely to report unprotected sex (76.4%), multiple sex-partners (87.1%), and the presence of an STI (64.2%) (17). Of the IDU registered with the Cooperative for Assistance and Relief Everywhere (CARE)-Bangladesh (3,900-4,400), an NGO, 55% were reported to be married. IDU are encouraged to bring their spouses to the drop-in center (DIC) for STI management, but there is no formal contact-tracing program.

The 5th and 7th round sero-surveillances found that IDU were at elevated risk of acquiring and transmitting HIV, also through unsafe sex practices. Very little is known about female IDU in Bangladesh, but anecdotal evidence suggests that they are hidden, and are very vulnerable to HIV through both their injection sharing and sexual risk behaviors. The special vulnerability of female IDU was highlighted by the 2006 ICDDR,B cohort study. Beyond their vulnerability to HIV through unsafe injection and sexual risk behavior, female injectors reported more anal sex and serial sex with multiple partners, as well as other hazardous experiences, such as being victims of sexual violence or being jailed (15). Once HIV enters this community, the female IDU are likely to bridge the epidemic to the general population. The 5th round sero-surveillance noted that heroin smokers also engaged in significant risk behaviors, in that more than one-third (34.4%) of them had also injected in the last 6 months, and almost all (96.3%) shared their syringes. In addition, many sexually active heroin smokers engaged in unprotected commercial sex (73.6% had sex with female CSW in the previous month, and condom use was only 3.8%) and had multiple sexual partners (the reported median number of partners was 4). Although the 7th round sero-surveillance indicated that many of the risk behaviors of heroin smokers had been significantly reduced, the still high level of needle sharing (72.6%) was a major concern.

CSW: There is a great deal of gender violence and inequality in Bangladesh society, which is largely male dominated, and women and girls are therefore at additional risk of contracting HIV. For many reasons, Bangladesh supports a large-scale commercial sex industry. The total number of CSW is unknown, but a 1995 estimate reported around 100,000 female CSW in the country (18), and the number may reach 150,000 in 2007. Unfortunately, relatively little has been reported on the levels of commercial sex outside Dhaka, although it is known that much of the sex with prostitutes is unprotected sex, and that most married men who patronize prostitutes continue to have sex with their wives (19). Khan and Arefeen (9) reported that police registration-of-prostitutes statistics show there is a significant number of prostitutes in all of the larger towns, includinh Dhaka, Chittagong, Khulna, Rajshahi, Mymensingh, and others. There are only 15 registered brothels, but hundreds operate as part of a hidden sex industry, and many of the prostitutes working in these brothels are treated inhumanely. All other sex-working venues, e.g., hotel-based sex work (HSW), street sex work (SSW), or residential-based sex work (RSW) are clearly illegal in Bangladesh. These CSW register their names with a magistrate, signing an affidavit that they are entering the profession of their own will, are over 18 years of age and have no alternative way of making a living. However, in reality they have often become CSW in response to poverty and other problems in their families. Police are allowed to raid brothels often in order to remove the women under 18 years or to search for criminals.

Female CSW from Bangladeshi cities close to India and Myanmar frequently cross borders to sell sex. From cities in the region labeled as Northwest-K1, about 70% of the female CSW had crossed into another country for sex work (20). A study of 867 female CSW in brothels in Kolkata, India, identified that nearly 20% were from Bangladesh (21). In addition, the estimated number of women and girls trafficked annually out of the country is 10,000-20,000. Some reports have indicated that 40,000 children from Bangladesh are involved in prostitution in Pakistan. There is also significant internal movement within the country to urban centers for the same purposes. The brothel-based sex work (BSW) and HSW in Bangladesh report an average of 18.8 and 44.0 clients per week, respectively, which is among the highest turnover of clients anywhere in Asia (22). Moreover, riskier forms of sexual intercourse are fairly common. Almost one in five CSW reported anal sex with new or regular clients in the past week, and, except among BSW, group sex was common, reported by 47-63% of all the CSW (14). Inconsistent condom use is also common, which increases the vulnerability of the workers to contracting HIV and other STDs. A study on BSW found that only 36% of sex acts had been protected by a condom during the last working day, and only 3.7% of female CSW had used condoms consistently during their last two working weeks (23). A study conducted among SSW ($n = 269$) in Dhaka found that overall, 84% were positive for at least one STI pathogen (24), and another study among BSW ($n = 439$) in four brothels found 67.4% were positive for at least one cervical and/or vaginal infection (25). The lower rate of condom use is a combined result of clients' dislike of condoms, lack of knowledge, low risk perceptions, and poor situational availability of condoms. Offering a condom to a client is a major trigger for violence, and contributed to around 36% of all the violence experienced by BSW (26). Safer sex practices are even more difficult for SSW, as the level of harassment is substantial. Accordingly, condom use is reported to be lower among SSW compared to other venues (27). The sero-surveillances conducted in 2003-2004 and 2006-2007 show there has been a marked recent increase in condom use among female CSW, but little change among male CSW and a remarkable decrease among MSM. However, the fact that many female CSW serve a large number of clients raises some doubt about how reliable their recall of condom use might be, and how consistent their condom use is.

Clients of CSW: The clients of female CSW include transport workers as truckers, dock workers, their helpers and cleaners, and rickshaw pullers; uniformed forces, young people, working children, women in domestic work or in the workplace setting and in particular female garment-workers, internal and international migrants, slum-dwellers, and tribal people. However, surveillances and individual studies have concentrated their efforts only on rickshaw pullers, truckers, slum dwellers, and students. A survey of sexual behaviors among men in rural populations found that one-fifth of all men and one-third of unmarried men reported paying for commercial sex at least once in their life-time (28) and a similar picture in urban areas was seen. An average 30-40% surveyed and sentinel sites reported buying sex in the previous year from female CSW with inconsistent condom use (<15%).

In the Northwest of Dhaka, over 60% also reported non-commercial sex, while 35-40% in Central and Southeast Dhaka did (11). A study in Dhaka (n = 388) found that 54% of the subjects (truck drivers/helpers) had relations with at least one commercial CSW in the past year, and their mean number of sexual partners in the previous year was 4.6; in addition, premarital and extramarital sex was common, often with CSW (29). Of these subjects, only 31% had used a condom, and most had used condoms only once or occasionally. However, the sample was not randomly recruited and the participants were from only one truck stand. The data were collected through self-reports in oral interviews, so responses may have been influenced by perceived social desirability. It is estimated that there are about 0.3 million rickshaw pullers in Dhaka City alone (30), but the actual number may be 0.7 million including workers from other cities who travel to Dhaka and often sex with the CSW there. Such migration patterns can lead to sex ratio imbalances in both cities and rural areas. Many of these rickshaw pullers have migrated from rural areas and have left their wives behind. In a study of 1,000 randomly chosen rickshaw pullers in Dhaka, more than 30% visited a brothel on a regular basis, and 22% had a history of STIs (31). Among the married rickshaw pullers, 35% had been practicing extramarital sex, and only 8% regularly used condoms. The 4th round sero-surveillance identified that only 4-15% of rickshaw pullers reported condom use during their last sex act, but students were among the three most common groups of clients seen by CSW.

MSM and transgender: MSM and hijra are often overlooked as a high-risk population for their infection activities in Bangladesh. The role of MSM in the spread and transmission of HIV has not been well studied, and is limited by a number of factors. Simple identification of a male as 'homosexual' overlooks many social and general issues that may contribute to MSM behavior (32). Also, male-to-male sex in Bangladesh is an offense under section 377 of the Bangladesh penal code (33). MSM in Bangladesh are at increased risk for HIV infection due to sexual behavior, including low condom use, association with IDU (5) and blood sales. The sero-surveillance data indicate that infection has reached significant proportions in certain high risk groups and may soon spread to other groups, especially MSM. Trends from other settings suggest that HIV will spread among these high risk groups before spreading to the general population. An NGO, as part of its community-based STI/HIV intervention, claimed that it reached a total of 1,454 MSM and male CSW between July 2000 and June 2001 (34). The 5th round sero-surveillance found around 45% of male CSW both from Central-A and Southeast-A of Dhaka City reported condom use in commercial sex with new clients in the past week. Khan et al. (35) have outlined the vulnerability of female sex partners of MSM. Half of the MSM surveyed in a port city in 2000 performed unsafe anal sex with females, including their wives. They often do not disclose their MSM practices to their female partners.

Transgender persons are traditional transvestites or transsexuals from the Indian subcontinent. Some are born phenotypically male, and some are said to have ambiguous genitalia. Traditionally, those who are born with ambiguous genitalia have their external genitalia removed surgically and become eunuchs. They wear women's clothing, and usually behave like women. An NGO working with this group estimated around 5,000 transgender live in Dhaka City alone (36). Most of them work as CSW and practice receptive anal sex (32). Transgender persons reported a very high average number of clients that they had unprotected sex with. In the 5th round surveillance, almost all transgender persons (99%) were reported to have sold sex in the last week, but only 17% of these reported condom use. In keeping with these data, transgender persons had the highest rate of active syphilis (10.4%) among all the groups in the 4th round sero-surveillance, and the proportion that reported condom use in the last sex act with clients (3.4%) in the 3rd round survey.

RESULTS AND DISCUSSION

Bangladesh is unique among the countries in South Asia in that it has most of the known risk factors for a large-scale HIV epidemic, but no evidence that such an epidemic is evolving. Many Bangladeshis explain this low level in terms of the predominantly Muslim composition of the society and Islam's teaching that sex should be confined to marriage. However, the exact reason why HIV infection is currently concentrated in Central A of Dhaka is unknown, but probable explanations include rural-urban migration patterns, the concentration of poverty and joblessness in the central city slums, and the greater availability of injecting drugs and a wider sharing network. Injecting drug use behaviors have been found throughout the country, but, HIV infections are not found at all sites. Therefore, the response has been focused mainly on where active infections have been documented. The epidemiology of HIV infection in other countries suggests that increasing rates of HIV in higher-risk populations can precede an epidemic in the general population. The available data from 7th round of national surveillances for HIV in Bangladesh represents the most complete information for high risk groups (Tables 1-4). It shows a fairly low, but rising, level of knowledge about HIV, but because the population is large, the rise in knowledge is as yet insufficient. Female CSW were the best informed, with 31% reporting correct knowledge, and IDU were the least well informed, with only 20% reporting correct knowledge. High population mobility within the country and beyond its borders has resulted in an increase in vulnerability to HIV and AIDS, particularly among young people. The average scores for the most-at-risk population groups included in the 7th round sero-surveillance was 25.9%, up from 17% in 2004, but a fairly low figure nonetheless. The prevalence of reproductive tract infections (RTIs) and STIs among females in the general population and among female CSW in Bangladesh is not well documented, although these infections are increasing tremendously. The link between HIV and other STDs is exacerbated in Bangladesh because of the paucity of effective and affordable treatment options and also because STIs and STDs are associated with a sense of shame and embarrassment; in addition, medication costs are a concern. The increase in chlamydial infection, which rarely shows clear symptoms, is also of grave concern. The increasing prevalence of HIV among IDU may predict a more general increase of HIV in the Bangladesh population over the next several years. Although Bangladesh is a low prevalence country for HIV/AIDS, all the factors that lead to rapid spread of infection and thus to an epidemic are present. These factors include high-risk behavior, lack of awareness, very mobile populations, and being surrounded by high HIV prevalent countries. Periodic surveillance of recognized high risk groups shows that HIV prevalence has been increasing steadily. In Dhaka City, HIV prevalence in one subset of a high risk group has crossed to

the level of a concentrated epidemic (7%). The high prevalence of sexual risk behaviors among IDU and CSW and their clients is alarming. Females are at risk of a major HIV epidemic from both infection sharing and sexual risk behavior, and sex workers who are also IDU appear especially vulnerable. Although a small increase in condom use and a reduction in syphilis have been seen among subsets of the most at-risk populations in recent years, these gains are clearly not sufficient to reduce the threat of a possible HIV epidemic. Once HIV is established in the risk groups, it will spread to the general population because the spread is determined by how risky people's sexual behavior is.

Estimations and projections of HIV/AIDS transmission up to the year 2020 in Bangladesh only through the risk groups are presented in Table 5 and Figures 3 and 4. The projections have shown a steady increase in the number of HIV/AIDS. The total number of HIV/AIDS among the entire population of Bangladesh may in fact be double that of the projected prevalence among the most-at-risk groups. All the populations among the risk groups have been considered in this estimation and projection. The average prevalence rates of the risk group populations were shown to be lower than the prevalence rates in Central Bangladesh. The reason for this is that in some cities, the HIV prevalence rates of the subsets of the most-at-risk groups in these cities are nearly zero. Moreover, the government along with a large number of NGOs have given top priority to working to prevent HIV in the risk groups and in the general population. To complement the efforts of the government, approximately 385 NGOs are actively involved with anti-HIV/AIDS work, primarily doing prevention. There is an NGO network of over 180 NGOs called the STI/AIDS Network. Their continued efforts in this regard are remarkable, and are the reason why the total number of HIV/AIDS is not comparatively high given the huge population of Bangladesh. Among the most-at-risk groups, IDU are the most vulnerable to HIV/AIDS, and the numbers of IDU affected have been gradually increasing, as is clearly shown in the curve i in Figure 3 and curve iii in Figure 4, followed by sex workers (curve ii in Figure 3 and curve ii in Figure 4), clients of sex workers (curve iii in Figure 3 and curve i in Figure 4), and MSM (curve iv in Figure 3 and curve iv in Figure 4). The Ø values for all the risk groups are positive, which means their transmission rates are increasing monotonically over time. However, the situation is more complex than these values indicate, because there is a surprisingly high level of commercial sex and intravenous drug use, and a significantly high level of STDs within the country. The results show that the most contributing factor is sex work among the wide population. The reason is people from rural areas who have commercial sex while in cities and towns will become infected, and a fraction of these persons will subsequently infect other partners in the rural population. Thus, Bangladesh adjoins the Asian region with severest AIDS epidemic.

The actual epidemiological picture of Bangladesh is incomplete due to the lack of published comprehensive serosurveillance data. Bangladesh is now at a critical moment, in that if actions are taken to control HIV transmission among

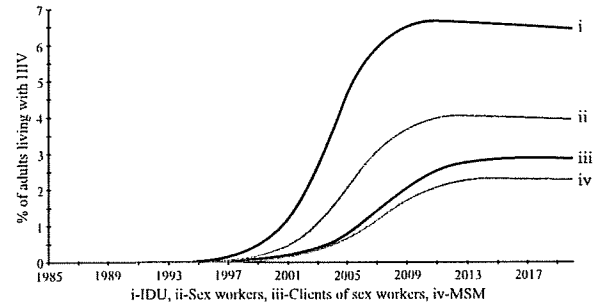


Fig. 3. Transmission rates of the most-at-risk groups for the populations up to 2020 in Bangladesh.

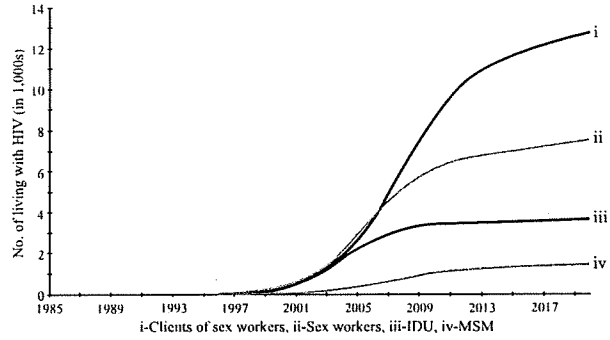


Fig. 4. Number of living with HIV of the most-at-risk groups for the populations up to 2020 in Bangladesh.

Table 5. HIV prevalence and rates among the most-at-risk groups for the populations of Bangladesh

Year	HIV of most risk group	Rate of IDU	HIV of IDU	Rate of MSM	HIV of MSM	Rate of clients CSW	HIV of clients CSW	Rate of CSW	HIV of CSW
2005	8,160	4.61	2,250	0.67	320	0.79	2,642	2.04	2,948
2006	10,602	5.40	2,668	0.91	445	1.07	3,684	2.59	3,805
2007	13,084	5.95	2,975	1.18	590	1.40	4,913	3.07	4,606
2008	15,453	6.33	3,200	1.46	743	1.75	6,235	3.45	5,276
2009	17,545	6.54	3,340	1.71	890	2.07	7,527	3.71	5,788
2010	19,277	6.62	3,420	1.92	1,019	2.34	8,680	3.88	6,158
2011	20,653	6.66	3,474	2.08	1,123	2.54	9,634	3.97	6,423
2012	21,695	6.65	3,498	2.18	1,204	2.69	10,378	4.02	6,614
2013	22,489	6.63	3,523	2.25	1,265	2.78	10,941	4.03	6,760
2014	23,082	6.58	3,531	2.28	1,310	2.83	11,362	4.03	6,878
2015	23,556	6.56	3,552	2.30	1,345	2.86	11,682	4.02	6,978
2016	23,945	6.52	3,567	2.30	1,373	2.87	11,936	4.00	7,070
2017	24,314	6.50	3,588	2.30	1,397	2.87	12,158	3.98	7,170
2018	24,669	6.49	3,615	2.29	1,419	2.86	12,357	3.97	7,278
2019	25,028	6.47	3,636	2.28	1,440	2.85	12,551	3.97	7,401
2020	25,388	6.48	3,676	2.27	1,462	2.84	12,735	3.96	7,515

Abbreviations are in Table 1.

the high risk groups, particularly IDU, a large-scale sexual epidemic may still be avoided. Female IDU are comparatively more vulnerable to HIV through their infection and sexual risk behaviors, and sex workers who are also drug users appear especially vulnerable because of their dual activity. In contrast, the prevalence of HIV among CSW has always been reported as being below 1%. The current low prevalence is the consequence of the failure to detect a more widespread epidemic. HIV is showing an increasing trend among recognized risk groups, and the high prevalence of risky behaviors by these groups could counterbalance the prevention efforts that have been implemented. For the most at risk groups, projects on risk behaviors, anthropological studies, epidemiological studies, and STI and virological studies need to be conducted among IDU, migrants, CSW, and other vulnerable groups including fishermen, smugglers, and ethnic minorities. To prevent a major epidemic from occurring, Bangladesh must implement a multi-faceted strategy. By concentraing on groups most vulnerable to infection, the onset of the epidemic can be prevented, or, if an epidemic begins, it can be prevented from escalating. A longer term generalized epidemic can be avoided by working with the general population, and by providing care and support to those already infected. It is critical for a comprehensive prevention program to be implemented that includes not only education but condom promotion. In addition, effective management of all STIs, a screening program for migrant workers, the contribution of both behavioral and serological components of HIV surveillance to cover the remaining high risk groups, with due consideration to the consistency of the surveillance indicators. Initiatives are also needed to develop pre-departure and post-departure programs for international migrants. Finally, increased coordination among intervening agencies would help ensure comprehensive prevention programs and equitable coverage. The range and quality of responses to HIV risk need further improvement. The expansion of surveillance to cover the remaining high-risk groups, and continuation of both components of the sero-surveillance, with the use of consistency of methodology, are important. Supportive care for HIV positive persons, more voluntary counseling and testing, and consideration of public health-oriented services for CSW are other pressing issues that need immediate attention. Steps such as these could help prevent the immense suffering and economic cost that high rates of HIV would bring to Bangladesh.

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Inhibition of HIV-1 replication by long-term treatment with a chimeric RNA containing shRNA and TAR decoy RNA

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ABSTRACT

Combinatorial therapies for the treatment of HIV-1 infection are effective for reducing patient viral loads and slowing the progression to AIDS. Our strategy was based on an anti-HIV-1 shRNA vector system in which HIV-1 vif-shRNA was fused to a decoy TAR RNA (mini-TAR RNA) to generate vif-shRNA-decoy TAR RNA under the control of the human U6 Pol III promoter. Upon expression in human cells, the RNA molecule was cleaved into its component parts, which inhibited HIV-1 replication in a synergistic manner. This chimeric RNA expressed a dual RNA moiety and greatly enhanced the inhibition of HIV-1 replication under the production of resistant virus by short interference RNA (siRNA) in long-term culture assays. We suggest that this technique provides a practical basis for the application of siRNA-based gene therapy in the treatment of HIV/AIDS.

1. Introduction

The emergence of multiple drug-resistant strains of HIV-1 has necessitated the development of alternative approaches for AIDS treatment (Cogoni et al., 1994). Unfortunately, chemotherapy adjuvants such as gene therapy are hampered by difficulties in delivering therapeutic genes to target cells. Lentiviral vectors, however, can be used for long-term and sustained gene expression and have the additional advantage of not requiring cell division to enter the cell nucleus.

RNA interference (RNAi) is a powerful tool for suppressing gene function and has generated much excitement in the scientific community (Baulcombe, 1996; Cogoni et al., 1994; Fire et al., 1998; Kennerdell and Carthew, 1998; Ngô et al., 1998). RNAi is triggered by small-interfering RNAs (siRNAs) that are processed from long double-stranded or hairpin precursors and become part of the RNA-induced silencing complex (Lipardi et al., 2001; Sijen et al., 2001). siRNAs are expressed from DNA templates silence gene expression as effectively as exogenously introduced synthetic siRNAs (Brummelkamp et al., 2002; Paddison et al., 2002; Paul et al., 2002; Zeng et al., 2002). The use of RNAi has been extended to differentiated cultured mammalian cells (Elbashir et al., 2001; Kretschmer-Kazemi and Sczakiel, 2003), and successfully inhibits the replication of pathogenic viruses in culture, including the human immunodeficiency virus (HIV) (Bitko and Barik, 2001; Coburn and Cullen, 2002; Gitlin et al., 2002; Jacque et al., 2002). A series of different RNA or multiple shRNA based-inhibitors were developed for use in a gene therapy-based treatment of HIV-1 infection (Anderson et al., 2007; Barnor et al., 2005; Ter Brake et al., 2006; Li et al., 2006), but the emergence of siRNA-escape variants following siRNA administration in long-term cultures has been reported (Boden et al., 2003; Das et al., 2004; Westerhout et al., 2005).

In the present study, we designed an anti-HIV short-hairpin RNA (shRNA) that encodes a cleavable HIV-1 virion infectivity factor (vif) shRNA-decoy *trans*-activation response region (mini-TAR RNA) (Banerjea et al., 2004; Hamma and Miller, 1999; Huq et al., 1999; Li et al., 2005; Selby et al., 1989). The chimeric RNA expressed a dual RNA moiety and greatly enhanced the inhibition of HIV-1 replication under the production of resistant virus. The decoy TAR RNA domain engaged the HIV-1 tat protein in a competitive interaction, thereby attenuating the HIV-1 transcriptional *trans*-activation process (Fulcher and Jans, 2003; Michienzi et al., 2002). The expressed

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HIV-1 vif siRNA domain-mediated post-transcriptional degradation of HIV-1 cognate genes to achieve a dual inhibitory effect on HIV replication.

2. Materials and methods

2.1. Construction of U6 expression plasmids and lentiviral-based vectors

Expression plasmids were constructed using standard techniques. Hairpin shRNA sequences chemically synthesized as two complementary DNA oligonucleotides (see Supplementary Table 1) were mixed in equimolar amounts, heated for 5 min at 95 °C, and then gradually cooled to room temperature in annealing buffer (10 mM Tris–HCl, 100 mM NaCl). The resultant duplex was ethanol-precipitated, and ligated into KpnI and BamHI cloning sites upstream of the U6 promoter (Lee et al., 2002) of pSV2neo (TAKARA, Otsu, Japan). The U6 vif-shRNA TAR vector encodes both HIV-1 vif-shRNA and decoy TAR RNA; the U6 vif-shRNA vector encodes HIV-1 vif; the U6 vif-shRNA Ran vector encodes random vif; the U6 TAR vector encodes HIV-1 TAR; the U6 MTAR vector encodes a mutated TAR loop; the U6 vif-shRNA Ran-MTAR vector encodes random vif and mutated TAR.

To construct the lentiviral vectors, the EcoRI fragment of the U6 vectors listed above containing the U6 promoter and the siRNA duplex was cloned into the EcoRI site of the lentiviral transfer vector (pCS-CDF-CG-PRE) (Miyoshi et al., 1998), generating the CS-vif-shRNA TAR and control transfer vectors.

2.2. Cell culture

Peripheral blood mononuclear cells (PBMCs): human pooled PBMCs were isolated from four healthy HIV-seronegative donors by Ficoll-Paque Plus separation (Amersham Biosciences, Buckinghamshire, UK). The pooled PBMCs were cultured for 24 h and half the culture volume was replaced with the same volume of PBMCs

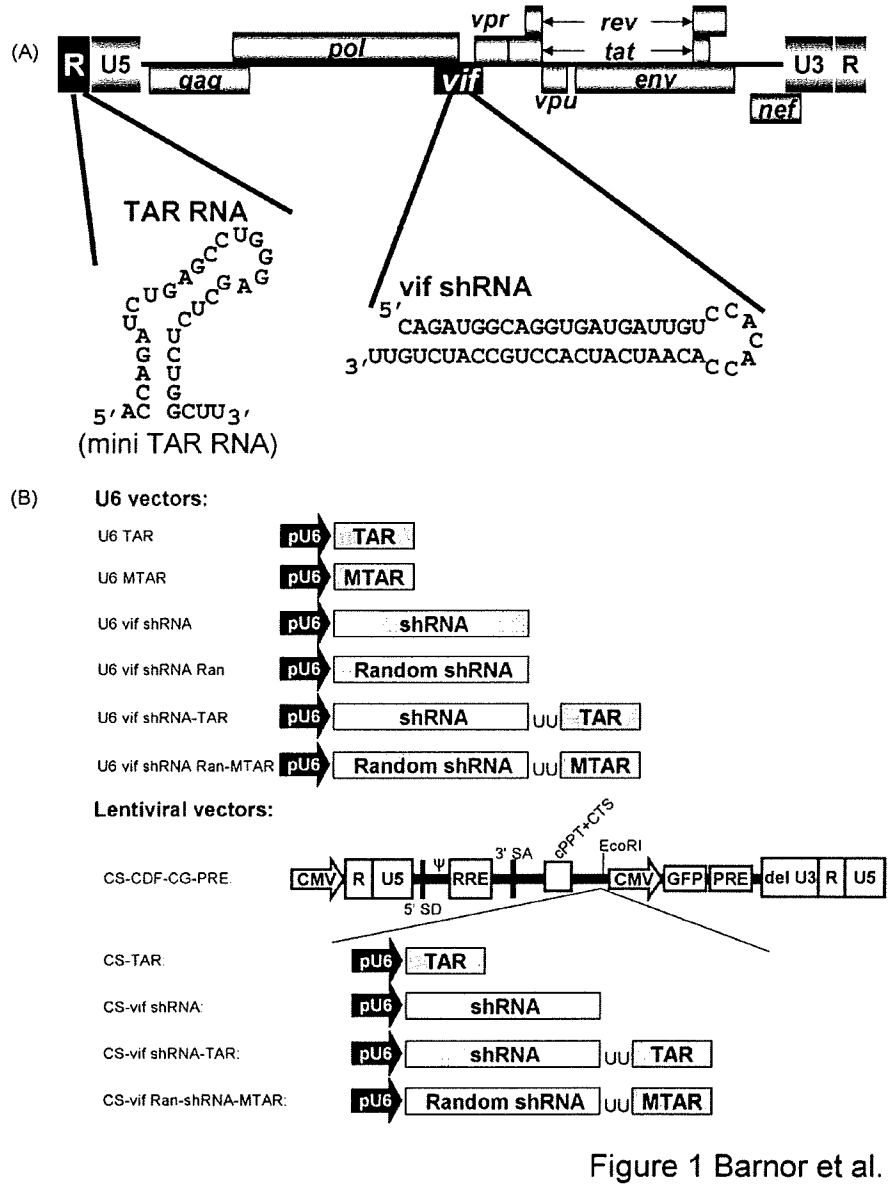

Figure 1 Barnor et al.

Fig. 1. Construction of second-generation shRNA expression vectors. (A) Schematic representation of HIV-1 genome showing decoy TAR RNA and vif-shRNA target sequences. (B) Schematic representation of human U6 promoter-driven U6-plasmid and CS-lentiviral vectors.

from a single donor (Vella et al., 1999). This process was repeated every 2 days for approximately 1 week before cells were used in the experiments. PBMCs, HeLa CD4$^+$, 293T, Jurkat, H9, and MT-4 cells were grown in either RPMI 1640 (Sigma, St. Louis, MO) or Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). All cultures were maintained at 37 °C under a 5% CO_2 atmosphere.

2.3. In vitro and intracellular Dicer cleavage assay

Purified vif-shRNA TAR dsRNA (60 μg) transcribed with T7-RNA polymerase using a BLOCK-iT RNAi TOPO Transcription Kit (Invitrogen, Carlsbad, CA) was cleaved in a 300-μl reaction volume according to the manufacturer's protocol (BLOCK-iT Dicer RNAi Kit; Invitrogen). Briefly, the dicing reaction was incubated at 37 °C for 20 h, and the transcript was added to non-transfected control cell lysate for normalization. The products were resolved on a 20% (w/v) polyacrylamide gel (Invitrogen). HeLa CD4$^+$ cells were transfected with 3 μg each vector DNA using Lipofectamine 2000 reagent (Invitrogen), and total RNA was extracted with Trizol according to the manufacturer's instructions (Invitrogen). Both 72 h post-transfection and T7-transcribed RNA were subjected to Northern blot analysis.

2.4. Northern blot analysis

Total RNA was extracted from 5×10^5 HeLa CD4$^+$ cells after transient transfection using Trizol reagent according to the manufacturer's instructions (Invitrogen), and 30 μg samples were loaded onto a 20% (w/v) polyacrylamide/8 M urea gel. After transfer to a Hybond-N^{TM} nylon membrane (GE Healthcare Bio-Sciences Corp., Piscataway, NJ), synthetic oligonucleotides complementary to the antisense strand of the vif-shRNA-decoy TAR RNA were used as probes. Hybridization was performed at 37 °C, followed by washing with 2× SSPE at 39 °C and 1× SSPE at 41 °C, prior to autoradiographic exposure.

2.5. Dose-dependent inhibition of HIV-1 replication

The indicated U6 vectors (0.1, 1, and 3 μg; as in Fig. 1B) were co-transfected with 0.2 μg HIV-1pNL4-3-EGFP into 3×10^5 HeLa CD4$^+$ cells. The HIV-1pNL4-3-EGFP infectious molecular clone encoding EGFP (Miura et al., 2001) was based on the previously described HIV-1pNL4-3 (Adachi et al., 1986). Cell-free culture supernatants were harvested and extracellular HIV-1 gag p24 antigen production levels were measured as an index of viral replication.

2.6. Down-regulation of target mRNA and inhibition of HIV-1 replication

Total RNA from HeLa CD4$^+$ cells co-transfected with 2 μg vector DNA and 0.2 μg HIV-1pNL4-3-EGFP was extracted with Trizol reagent after 72 h of culture. The RNA content was examined using primer sets (forward: 5′-AAG TAG TGT GTG CCC GTC TGT TG-3′ and reverse: 5′-CTA GGA TCT ACT GGC TCC ATT TCT TGC-3′) that allowed for the detection of HIV-1 vif viral RNA, and for TAR RNA (forward: 5′-GCA ATG ATT GTC GTA ATT GC-3′ and reverse: 5′-CTT GCT CAG TAA GAA TTT TCG TC-3′). HIV-1 gag p24 antigen production levels were then measured in the cell-free harvested culture supernatants using a fully automated chemiluminescent enzyme immunoassay system (Fujirebio, Tokyo, Japan) (Sakai et al., 1999).

2.7. Lentivirus preparation

293T cells were co-transfected with 15 μg transfer vector construct, 15 μg helper constructs coding for Gag-Pol (pMDLg/p.RRE), 5 μg Rev-expressing construct pRSV-Rev, and 5 μg VSV-G expressing construct pMD.G, using the calcium phosphate-precipitation method (Stegmeier et al., 2005). Supernatants were harvested 72 h post-transfection, filtered through a 0.45 μm filter disc, and concentrated 100-fold by centrifugation at $6000 \times g$ overnight. The resultant viral pellet was resuspended in serum- and antibiotic-free RPMI medium and stored at −80 °C until use. To determine the viral titer, 5×10^5 293T cells were transduced with the prepared viral stock, and the number of EGFP-positive cells was counted after 72 h culture using flow cytometric analysis (Ducrest et al., 2002).

2.8. Transduction of PBMCs and H9 cells

Human PBMCs (1×10^6), H9 cells (2×10^5), and Jurkat cells (2×10^5) were seeded in 12-well plates in 1 ml culture medium. Cells were transduced with the CS-vif-shRNA TAR and control lentiviral vectors at a multiplicity of infection (MOI) of 20 in the presence of 4 μg/ml polybrene. After incubation at 37 °C for 8–16 h, the medium was removed before the HIV-1 challenge was initiated.

2.9. IFN-β ELISA

Human IFN-β was detected in culture supernatants using a Human Interferon Beta (Hu-IFN-β) ELISA Kit (PBL Biomedical Laboratories, Piscataway, NJ) following the manufacturer's instructions. For control IFN production, 1 μg poly I:C was transfected into PBMCs with Lipofectamine 2000 according to the manufacturer's instructions. Transduction of lentiviral vectors is described above. Culture supernatants were assayed 2 days after transfection of poly I:C or transduction of lentiviral vectors.

2.10. Generation of viruses

To generate HIV-1 viruses, the HIV-1 infectious molecular cloned plasmid vector (HIV-1pNL4-3) was transfected (3 μg DNA) into 24-h seeded HeLa CD4$^+$ cells (5×10^5) using Lipofectamine 2000 according to the manufacturer's instructions. The culture was incubated at 37 °C for 72 h, then harvested and the cells pelleted by centrifugation to produce the cell-free supernatant yielding the HIV-1_{NL4-3} virus, which was aliquoted and stored at −80 °C. HIV-$1_{NL4-3-vif-mut}$ virus was generated from the experiment shown in Fig. 4B. Briefly, PBMCs transduced in the presence of 4 μg/ml polybrene with lentivirus-mediated vif-shRNA at an MOI of 20 and challenged with HIV-1_{NL4-3} virus at an MOI of 0.01 were cultured for 9 weeks at 37 °C. At week 6, harvested supernatant showing a vif mutation virus HIV-$1_{NL4-3-vif-mut}$ (siRNA vif target 5049-CAGATGGCAGGTGATGATTGT-5069; vif-shRNA/3 weeks post-infection, AGG-TGGCGA—ATGATTAT: 5 nt substitutions and 4 deletions), was titered and stocked at −80 °C and later used as the HIV-$1_{NL4-3-vif-mut}$ virus.

2.11. HIV-1 challenge and long-term culture assay

After transduction, PBMCs, H9 cells, and Jurkat cells expressing the transgenes were challenged with HIV-1_{NL4-3} at an MOI of 0.01. Following infection, the cells were washed three times with phosphate-buffered saline (PBS) and resuspended in growth medium. Mock infection was performed under the same conditions, except that the supernatants were generated from control/vector-transduced cells. One-half of the culture volume was harvested and replaced with an equal volume of culture medium at regular intervals. The harvested culture was centrifuged and the cell-free

medium used for HIV-1 gag p24 antigen quantification and viral RNA extraction, while the pellet was used for cell viability counts and FACS analysis of EGFP expression as a marker of transgene expression.

2.12. Flow cytometry analysis of long-term EGFP expression

One-half volume of culture was harvested 2, 4, 6, and 8 weeks post-challenge, pelleted, washed twice in PBS, and resuspended in 1% formaldehyde. FACS analysis was performed using the FACSCalibur and *CELLQUEST* software (BD Sciences, San Jose, CA).

2.13. Genotypic sequence analysis of the vif siRNA target region of HIV-1_{NL4-3} and rechallenge of wild-type vif-shRNA TAR expressing cells

Viral RNA from HIV-1_{NL4-3}-challenged CS-vif-shRNA TAR or CS-vif-shRNA-transduced cultures was analyzed for siRNA-mediated mutations in the vif-shRNA target region at weeks 2, 4, 6, and 8, as described previously (Sijen et al., 2001). Viral RNA was isolated from the cell-free culture supernatant using a QIAamp viral RNA kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. Viral RNA (5 μl) was used in a reverse transcription-polymerase chain reaction (PCR) reaction containing Powerscript reverse transcriptase (Clontech, Mountain View, CA), 1 μM each of the deoxynucleotide triphosphates, 1× first-strand buffer (Clontech), 200 ng random hexamer (Promega, Madison, WI), and 10 U RNasin (Promega). Reverse transcription was performed at 42 °C for 1 h, followed by heat inactivation of the reverse transcriptase enzyme at 70 °C for 15 min. cDNA (2 μl) was added to a 48-μl PCR mixture containing 1× Qiagen Taq PCR buffer, 1.5 mM $MgCl_2$, 20 pmol sense primer vif F: (5′-ATG GAA AAC AGA TGG CAG GTG AT-3′), and antisense primer vif R: (5′-CTA GTG TCC ATT CAT TGT ATG GCT-3′), 1 mM each of the deoxynucleotide triphosphates, and 2.5 U Taq polymerase (Qiagen). PCR was performed in a gradient PCR thermal cycler (Astec, Fukuoka, Japan) using the following thermal program: 1 cycle (95 °C for 1 min), 35 cycles (95 °C for 15 s, 58 °C for 30 s, and 72 °C for 30), and 1 cycle (72 °C for 5 min). The PCR product was fractionated and analyzed on a 1% SeaKem gel, and purified using a QIAEX II gel extraction kit (Qiagen). Nucleotide cycle sequencing was performed using dye-labeled terminator chemistry.

PBMCs stably expressing vif-shRNA-decoy TAR RNA, vif-shRNA, decoy TAR RNA, and the control (PBMCs, U6-ter) were challenged with either wild-type virus HIV-1_{NL4-3} or mutant virus HIV-$1_{NL4-3\text{-}vif\text{-}mut}$. Human PBMCs ($1 \times 10^6$) were infected with 20 ng p24 of each virus. Following infection, the cells were washed three times with PBS and resuspended in growth medium. The time-course of the infection was monitored over a 5-week period by HIV-1 gag p24 ELISA.

2.14. Statistical analysis

Statistical analysis was performed using a one-tailed Student's *t*-test. A *p*-value of less than 0.05 was considered significant. Data were based on means ± standard error (SE) of three separate experiments performed in duplicate.

3. Results

3.1. Construction of U6 expression plasmids and lentiviral-based vectors, and Dicer cleavage assay

We constructed vectors that specifically target the vif and TAR sequences of HIV-1 (Fig. 1A): the pSV2neo-U6-plasmids (U6) and pCS-CDF-CG-PRE-based lentiviral vectors (CS) (Fig. 1B), and

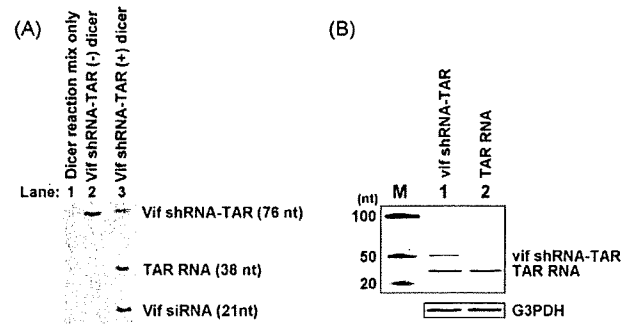

Fig. 2. Predicted secondary RNA structure, expression, and cleavage. (A) *In vitro* cleavage of RNA substrate. Northern blot analysis of T7-RNA polymerase-transcribed vif-shRNA-decoy TAR RNA (60 μg) treated with human recombinant Dicer. Lane 1: negative control without Dicer or sample; lane 2: vif-shRNA TAR RNA without Dicer detected with ^{32}P tagged vif-shRNA and TAR probe; lane 3: vif-shRNA TAR RNA with Dicer detected with ^{32}P tagged vif-shRNA and TAR probe. (B) Intracellular expression of vector RNA. Intracellular cleavage of chimeric RNA (vif-shRNA TAR) shown by Northern blot analysis of total RNA extracted 72 h post-transfection from HeLa CD4$^+$ cells. M: RNA size marker; lane 1: vif-shRNA TAR RNA detected with ^{32}P tagged TAR probe; lane 2: expressed decoy TAR RNA as an internal control marker detected with ^{32}P tagged TAR probe. G3PDH was used as an internal control and loading standard in both experiments.

analyzed the predicted RNA secondary structure of the vif-shRNA-decoy TAR RNA molecule using GENETYX software. Intracellular expression of U6 vif-shRNA TAR and control vector RNAs (U6 vif-shRNA, U6 TAR, U6 vif-shRNA Ran, U6 MTAR, and U6 vif-shRNA Ran-MTAR) was confirmed by Northern blot analysis after lipid transfection of HeLa CD4$^+$ cells (data not shown).

The RNAi mechanism is closely linked with the activity of the endogenous RNase III-like enzyme, Dicer (Kawasaki and Taira, 2003). To evaluate the cleavage efficacy of the vif-shRNA TAR RNA molecule, we incubated the transcribed substrate with human recombinant Dicer and subjected it to Northern blot analysis. This revealed high cleavage efficacy compared with the control non-Dicer cleaved vif-shRNA TAR RNA product (Fig. 2A). Furthermore, we investigated the *in vivo* processing of vif-shRNA-decoy TAR RNA into siRNAs and decoy TAR RNAs by endogenous Dicer in transfected HeLa CD4$^+$ cells. Northern blot analysis revealed that the vif-shRNA TAR RNA molecule was cleaved (Fig. 2B). However, the function as a decoy TAR RNA in chimeric RNAs was demonstrated by vif-shRNA-decoy TAR RNA present in the nucleus.

3.2. Inhibition of HIV-1 gene expression by shRNA and decoy RNA

To explore the dose-dependent inhibitory efficacy of the vif-shRNA TAR RNA molecule on HIV-1 replication, HIV-1 gag p24 antigen levels were measured in HeLa CD4$^+$ cells that were co-transfected with U6-ter, U6 vif-shRNA TAR RNA, U6 vif-shRNA, U6 vif-shRNA Ran, U6 TAR or U6 vif-shRNA Ran MTAR. U6 vif-shRNA TAR transfection inhibited HIV-1 replication in a dose-dependent manner with a greater than 90% maximum inhibitory efficacy for 72 h (Fig. 3A). Both U6 vif-shRNA TAR RNA and U6 vif-shRNA generated almost equally high inhibition. Furthermore, the level of enhanced green fluorescent protein (EGFP) expression, used as an index of replication, was notably reduced in transfected HeLa CD4$^+$ cells (Fig. 3B).

3.3. Long-term inhibition of HIV-1 replication by lentiviral vector-mediated shRNA-decoy RNA

Lentiviral (CS-vector) versions of the plasmid U6 vectors (Fig. 1B) were generated to enhance the delivery and durability of the RNA chimera by utilizing their ability to transduce non-dividing T-cells

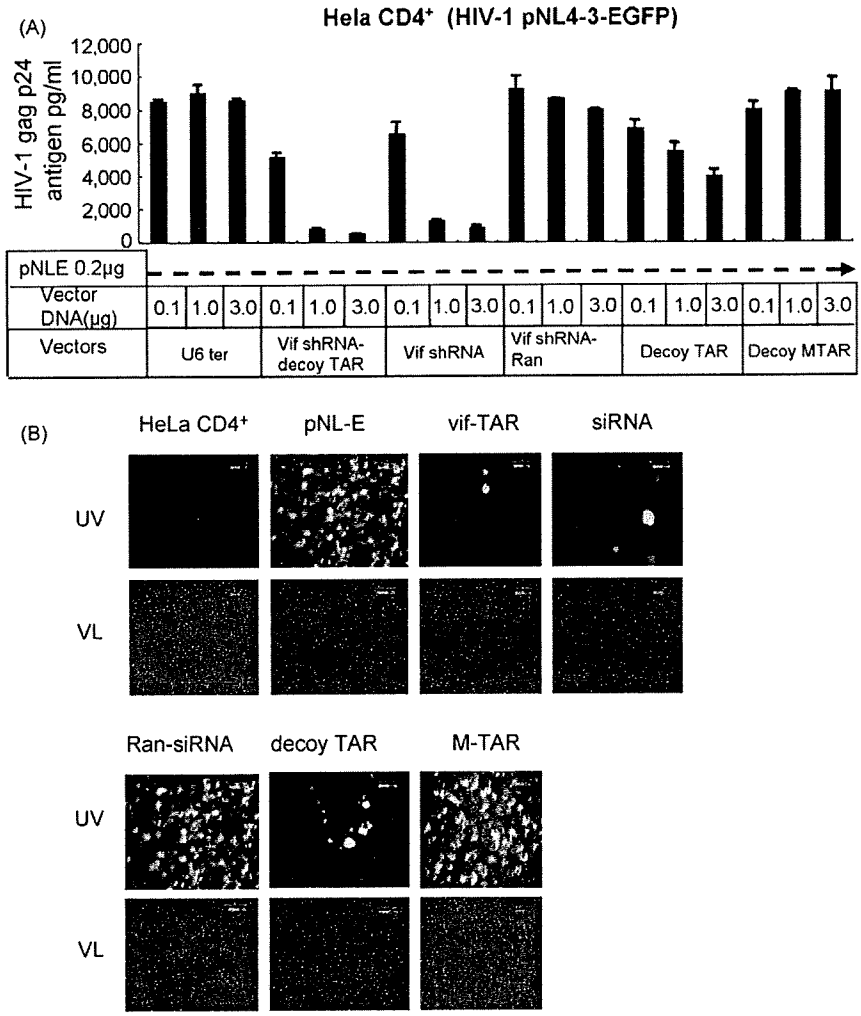

Fig. 3. Anti-viral efficacy of vector constructs. (A) HeLa CD4+ cells co-transfected with different concentrations of indicated U6 vectors and 0.2 μg HIV-1 pNLE4-3 were tested for HIV-1 gag p24 antigen expression using an automated ELISA system. Data represent mean values ± standard deviation (SD) of three independent experiments. (B) Down-regulation of reporter gene expression in HeLa CD4+ cells co-transfected with indicated U6 vectors and HIV-1 pNLE4-3 was examined under ultraviolet light (UV) and visible light (VL) to evaluate EGFP expression as an index of down-regulation of HIV-1 replication.

(Huq et al., 1999). Peripheral blood mononuclear cells (PBMCs), H9 cells, and Jurkat cells were stably transduced with these vectors. To determine whether vif-shRNA TAR was immunostimulatory, we transduced PBMCs with a lentiviral vector (CS-vif-shRNA TAR). An enzyme-linked immunosorbent assay (ELISA) for interferon (IFN)-β protein was performed on cell supernatants. IFN-β protein was not detected in supernatants from control cells or parental CS-CDF-CG-PRE (CS-blank) and CS-vif-shRNA TAR (CS-vif-TAR) transduced cells (Fig. 4A). These transduced cells were challenged with HIV-1_{NL4-3}, and HIV-1 gag p24 antigen levels were measured as an index of viral replication or inhibition by the expressed transgenes. The siRNA related-escape mutant phenomenon was observed at 3 weeks in the transduced PBMCs, as indicated by the virus breakthrough effect (Fig. 4B), compared with 4 weeks in H9 cells (Fig. 4C). In both PBMCs and H9 cells, CS-vif-shRNA TAR expressed RNA-mediated stable inhibition of HIV-1 replication was observed with the RNAi-resistant virus at 9 weeks (Fig. 4B and C). Similar inhibition levels were observed in both transduced cell types expressing the TAR RNA. A steady increase in viral expression was maintained until week 4, then fluctuations in inhibition efficacy were observed from week 4 to 9 in the H9 cells (Fig. 4C), and from week 5 to 9 in the PBMCs (Fig. 4B). Furthermore, to determine whether HIV-1 was down-regulated by APOBEC3G, APOBEC3G-defective Jurkat cells transduced with CS-vif-shRNA TAR were tested for HIV-1 gag p24 antigen expression. Similar results were observed in the APOBEC3G-expressing H9 cells (Fig. 4C) and APOBEC3G-defective Jurkat cells (Fig. 4D). To quantitatively estimate the duration of gene expression, we performed a time-course experiment that revealed EGFP expression at weeks 2, 4, 6, and 8 in both transduced PBMCs and H9 cells. EGFP expression persisted up to week 8 (Fig. 4E).

3.4. *RNAi-resistant HIV-1 variants*

siRNAs targeted to HIV genes in long-term stably expressing cultures give rise to escape mutants (Boden et al., 2003; Das et al., 2004; Westerhout et al., 2005). We therefore investigated the sudden upsurge in viral replication in cultures expressing vif-shRNA. Sequence analysis revealed that both cultures expressing vif-shRNA alone and vif-shRNA TAR RNA showed resistance against vif-shRNA (Fig. 5A). The vif-shRNA TAR, however, effectively inhibited 98% of HIV-1 replication at 9 weeks. Most surprisingly, there was an emergence of RNAi-resistant viruses that contained nucleotide substitutions or deletions in or near the shRNA-vif target sequence. Partial substitutes or deletions were observed in six cultures at 2

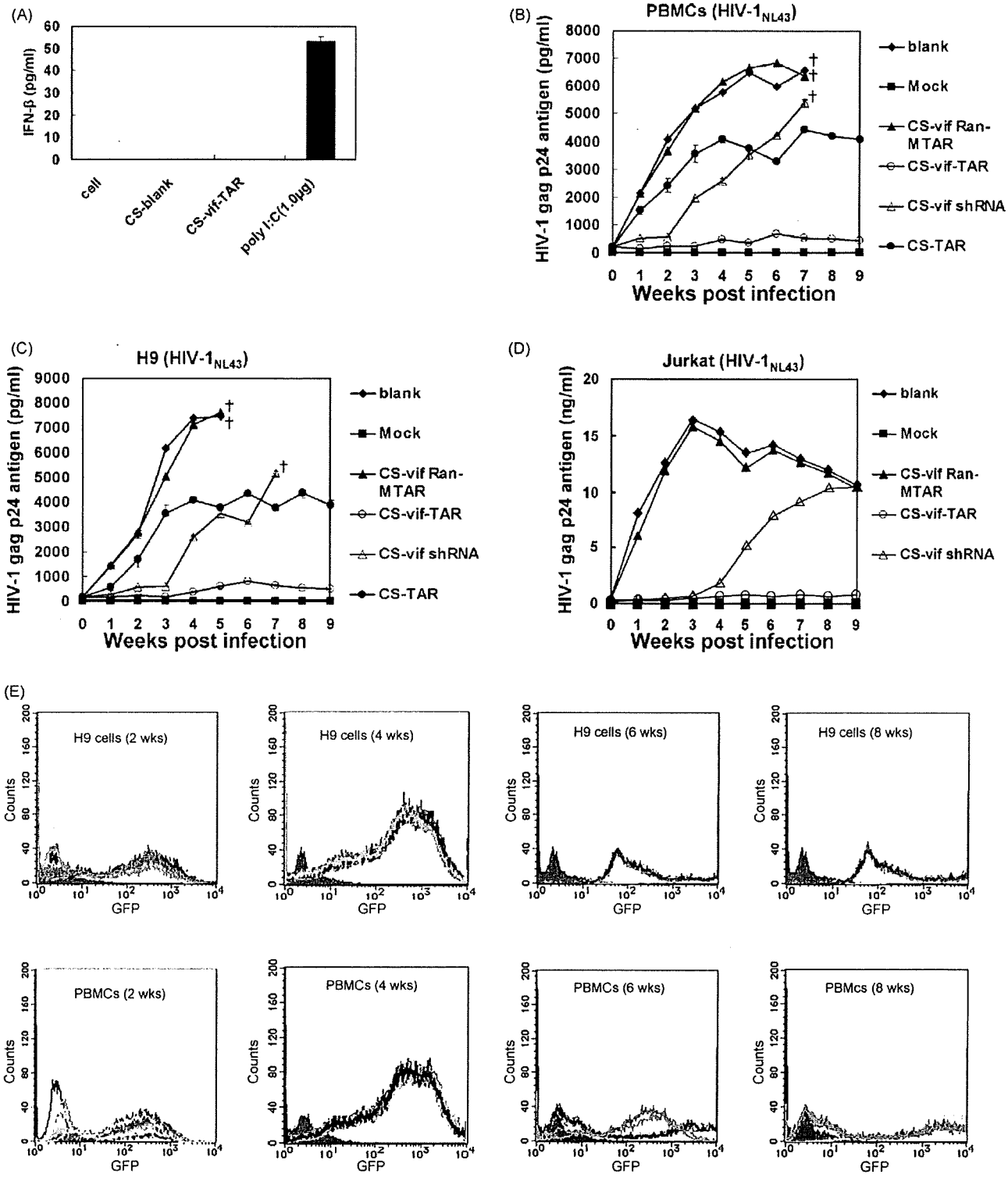

Fig. 4. Side effect and long-term activity of lentivirus-mediated vif-shRNA TAR RNA. (A) IFN-β ELISA was performed on supernatants. Data are from triplicate experiments. (B) Long-term inhibition of HIV-1 replication in PBMCs. HIV-1 gag p24 antigen expression was measured during the 9-week culture of PBMCs transduced with indicated CS-lentiviruses (MOI 20) and challenged with HIV-1_{NL4-3} (MOI 0.01). Data are from duplicate experiments. HIV-1 gag p24 antigen expression was measured during the 9-week culture of H9 (C) and Jurkat (D) cells under same experimental conditions as described for (B). (E) Long-term expression of transgenic EGFP expression in PBMCs and in H9 cells expressing vector transgenes was examined by FACS analysis using *CELLQUEST* software. Data are from duplicate experiments.

(A)

Vector	Week	Clone	siRNA-vif target	Nucleotide substitution / deletion
			5049 5069	
HIV-1 $_{NL4-3}$			CAGATGGCAGGTGATGATTGT	
shRNA	2	#1	CAGAGGACAGATGGAACAAGC	10 nt substitutions
	2	#2	CAGAGGACAGATGGAACAAGC	10 nt substitutions
	2	#3	CCTTGGGCAGGTGATGATTGT	4 nt substitutions
vif shRNA-decoy TAR	2	#1	CAGATGGCAGGTGATGATTGT	
	4	#1	CAGAGGACAGATTAACAAGCC	10 nt substitutions
	4	#2	TATCAAGCAGGACATAAGCAA	11 nt substitutions
	4	#3	CAGAGGACAGATGGAACA-GC	9 nt substitutions and 1 deletion

(B)

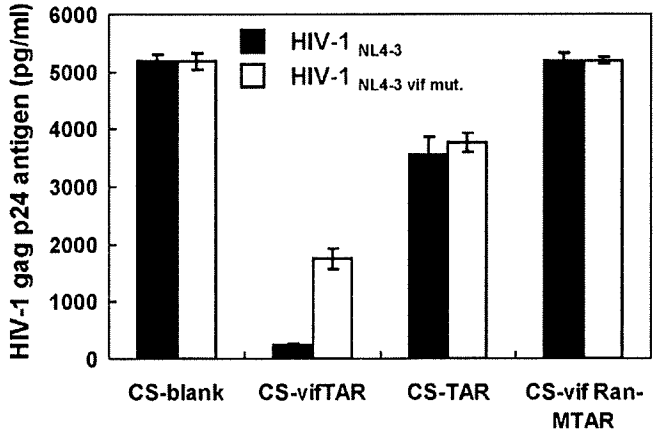

Fig. 5. HIV-1 escape variants that resist vif siRNA inhibition. (A) Genotype sequence analysis revealed siRNA-mediated mutations in vif-shRNA target site (nucleotides 5049–5069) of HIV-1_{NL4-3} in RNA extracted from vif-shRNA and vif-shRNA TAR RNA-expressing culture supernatants. Day at which escape variants were sequenced is indicated. Deletions are shown as dashes, substitutions are underlined and in bold. (B) PBMCs stably expressing vif-shRNA-decoy TAR RNA, vif Ran-shRNA-decoy MTAR RNA, and decoy TAR RNA cells infected with mutant virus HIV-$1_{NL4-3-vif-mut}$ and wild-type virus HIV-1_{NL4-3} over a 3-week period. Viral challenge of vif-shRNA-TAR RNA-expressing cells with wild-type virus HIV-1_{NL4-3} and mutant virus HIV-$1_{NL4-3-vif-mut}$ resulted in 98% and 68% inhibition of HIV-1 replication. Decoy TAR RNA alone showed the low inhibition of both HIV-$1_{NL4-3\,3}$ and HIV-$1_{NL4-3-vif-mut}$ infections. Viral challenge of the control, vif Ran-shRNA-decoy MTAR RNA-expressing cells with HIV-$1_{NL4-3-vif-mut}$ and HIV-1_{NL4-3} did not result in inhibition of HIV-1 replication. Data are from duplicate experiments.

weeks (vif-shRNA) and 4 weeks (vif-TAR shRNA). No mutations in the *tat* sequence were observed (data not shown).

To determine whether the viral escape mutant was indeed resistant to vif-shRNA, we took PBMCs stably expressing vif-shRNA-decoy TAR RNA, vif Ran-shRNA-decoy MTAR RNA, and decoy TAR RNA and infected them with the evolved HIV-$1_{NL4-3-vif-mut}$ and wild-type HIV-1_{NL4-3}. HIV-1_{NL4-3} caused 98% inhibition of HIV-1 replication in the vif-shRNA-decoy TAR RNA-expressing PBMCs at 3 weeks (Fig. 5B), whereas 68% inhibition was observed from mutant virus HIV-$1_{NL4-3-vif-mut}$ at the same time period. Further, decoy TAR RNA alone demonstrated 37% inhibition of HIV-1 replication, and its inhibitory effect was essentially due to the decoy TAR RNA. By contrast, viral challenge of the control, vif-Ran-shRNA-MTAR expressing PBMCs with HIV-$1_{NL4-3-vif-mut}$ and HIV-1_{NL4-3} did not result in the suppression of viral replication. These results demonstrate that the efficiency of siRNA-binding to target RNA can be diminished by nucleotide substitutions or deletions in the target sequence. These mutations presumably induce an alternative secondary structure in the RNA genome that reduces the efficiency of RNAi (Westerhout et al., 2005).

4. Discussion

RNAi is a potent inhibition technique that shows great promise for the treatment of HIV/AIDS. The sensitivity of the target region to RNAi, however, can lead to the emergence of RNAi-resistant HIV mutants (Boden et al., 2003; Das et al., 2004; Westerhout et al., 2005). Sequencing of emerging RNA-resistant viruses previously revealed alterations in the *nef* sequence; in some cases the siRNA recognition site contained several nucleotide substitutions that disrupted base-pairing and in other cases the target was deleted (Das et al., 2004). Analogous to the current clinical use of combinations of anti-viral drugs that target reverse transcriptase and protease enzymes, we propose that a combination of different therapeutic RNAs inhibiting multiple steps in the viral life cycle might be the most efficacious way to treat this infection in a gene therapy setting.

We investigated the intracellular processing of vif-shRNA-decoy TAR RNA into siRNAs and decoy TAR RNAs by endogenous Dicer in transfected HeLa $CD4^+$ cells. The vif-shRNA TAR RNA molecule was cleaved at 72 h (Fig. 2B). The first step in the HIV-1 inhibitory effect of these RNA molecules may arise from the decoy TAR RNA moiety of intranuclear RNA. Subsequently, the cytoplasmic vif siRNA cleavage product is expected to exert anti-HIV-1 activity by enhancing the inhibition of HIV-1 replication in infected cells. In this study, we demonstrated that each of the therapeutic RNA agents, vif-shRNA and decoy TAR RNA, and the vif-shRNA-decoy TAR RNA, had anti-HIV-1 activity (Fig. 3). On the other hand, decoy TAR RNA demonstrated a slightly reduced inhibitory effect on HIV-1 replication compared with vif-shRNA transfected cells. The vif-shRNA-decoy TAR RNA construct was effective for co-suppression of HIV-1 replication. As transfected shRNA plasmid vectors are likely to have only a transient effect, it will be necessary to use methodologies that induce constitutive expression in target cells to produce a sustained effect. To this end, the use of retroviral or lentiviral vectors to deliver shRNAs into cells would be ideal. Such a goal was accomplished in recent studies of lentiviral transduction of anti-siRNA into hematopoietic stem cells from which HIV-1-resistant T-cells and macrophages were derived (Hamma and Miller, 1999; Li et al., 2005).

We demonstrated that these inhibitors act as Pol III expression units within the lentiviral vector backbone. In both PBMCs and H9 cells, stably expressed CS-vif-shRNA TAR RNA inhibited HIV-1 replication with an RNAi-resistant virus at 9 weeks (Fig. 4B and C). Hence, siRNAs targeted to HIV genes in long-term stably expressing cells give rise to escape mutants, but RNAi-resistant HIV-1 was suppressed by decoy TAR RNA. There was no difference in 9-week p24 antigen suppression levels between transduced H9 cells (Fig. 4C). Moreover, a similar inhibitory effect of CS-vif-shRNA TAR RNA was observed in APOBEC3G-defective Jurkat cells (Fig. 4D). These results suggest that APOBEC3G had no effect in this vif-shRNA TAR RNA study. Further, IFN-α protein was not detected in the supernatant from control cells or CS-vif-shRNA TAR-transduced cells (Fig. 4A). This supports previous findings by Robbins et al. who demonstrated that IFN-β was not induced in lentiviral vector-transduced $CD34^+$ progenitor cells (Robbins et al., 2006).

Despite the inhibitory action of the single vif-shRNA, the siRNA related-escape mutant phenomenon was observed 3 weeks in transduced PBMCs as indicated by the virus breakthrough effect (Fig. 4B), compared with 4 weeks in H9 cells (Fig. 4C). Most surprisingly, RNAi-resistant viruses emerged that contained nucleotide substitutions or deletions in or near the shRNA-vif target sequence (Fig. 5A). The efficiency of RNAi-mediated inhibition depends on the efficiency of siRNA-binding to the target RNA. This interaction can be diminished by nucleotide substitutions or deletions in the target sequence that cause a mismatch with the siRNA or by mutations that induce a secondary RNA structure in which the target sequence is occluded (Westerhout et al., 2005). Furthermore, viral challenge of vif-shRNA TAR-expressing cells with mutant virus $HIV\text{-}1_{NL4\text{-}3\text{-}vif\text{-}mut}$ resulted in 68% suppression of HIV-1 replication (Fig. 5B), but cells with only decoy TAR RNA induced a low level of HIV-1 inhibition. The addition of the stem and hairpin loop region to the decoy TAR RNA sufficiently stabilized the structure of the decoy RNA to increase its HIV-1 inhibiting activity.

Decoy TAR RNA function in chimeric RNA is demonstrated by the interaction of vif-shRNA-decoy TAR RNA with HIV-1 tat protein present in the nucleus. Non-interacting vif-shRNA-decoy TAR RNA was exported to the cytoplasm, so the decoy RNA appears to contribute much less to the overall inhibition than the shRNA. Even so, decoy RNA activity was immediately restored by incorporating the TAR sequences into the shRNA-decoy RNA. We did not, however, observe any mutations in the *tat* sequence (data not shown). There was no difference in p24 antigen suppression levels at 9 weeks in either of the transduced H9 cells.

In conclusion, this study provides strong evidence that the development of a combination gene therapy strategy could have therapeutic importance for the delivery of multiple genes and the enhancement of inhibitory efficacy. The advantage of the lentiviral vector is that the anti-viral RNAs, shRNA and RNA decoy inhibit HIV-1 via different mechanisms. Future studies and improvements are needed to enhance the potential of this strategy as a novel method for siRNA-based HIV-1 gene therapy in the treatment of HIV/AIDS.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2009.04.008.

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