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GENOTYPING OF HUMAN PAPPILOMAVIRUS IN CERVICAL PRECANCEROUS LESION AND SQUAMOUS CELL CARCINOMA AT DR. SOETOMO HOSPITAL, SURABAYA, INDONESIA

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Abstract

Background: Cervical cancer caused by human papilloma virus (HPV), is the second most common cancer for women. This cancer is distributed worldwide, with ~80% of cases are found in the developing countries. In Indonesia, data of HPV genotypes are still limited and do not represent all regions of the country. Thus, here we report genotyping of HPV samples collected from the Dr. Soetomo Hospital Surabaya Indonesia patients, in 2013.
Materials and Method: A cross sectional study was performed using 68 paraffin blocks of low-grade squamous intraepithelial lesion (LSIL), high-grade squamous intraepithelial lesion (HSIL), and squamous cell carcinoma (SCC) cervix.
Result: This study showed that HPV genotypes found in LSIL samples are HPV 16, 18, 6/33 or 68/72. Furthermore, those in HSIL are HPV 16, 18, 52, 59, 67, 6/18, 6/45, 16/67, 26/61, or 52/67, while in SCC are HPV 16, 18, 45, 52, 56, 16/18 or 16/45. Single-genotype infection, *i.e.* by HPV 16, 18, 45, 52, 56, 59, or 67, was observed in 86.77% (59/68) of samples, whereas multiple-genotype infections, *i.e.* by HPV 6/18, 6/33, 6/45, 16/18, 16/45, 16/67, 26/61, 52/67, or 68/72, was found in 13.23% (9/68) of the samples.
Conclutions: The mostly HPV genotype identified in this study is HPV 16 (62.68%), then followed by HPV 18 (20.9%), HPV 45 (5.97%), 52 (5.97%), and 67 (4.48%). HPV 16 and 18 have used as vaccine, and HPV 45 has cross reaction with HPV 18, then HPV 52 and 67 should be considered as the second-generation HPV vaccines.

Keywords: Human Papillomavirus, squamous cell carcinoma, low-grade squamous intraepithelial lesion, high-grade squamous intraepithelial lesion

Introduction

Although cervical cancer (CC) can be potentially prevented, it remains a serious threat to women in a developing country like Indonesia. In 2008, WHO reported that ~79.14 million women in Indonesia (~35% of population) are at a risk for this cancer. Reports from WHO/ICO (2010) showed that annually ~13,000 new CC cases are diagnosed and ~7,500 deaths caused by this disease are observed. According to histopathological data from the Cancer Registration Agency of the Association of Indonesia Pathological Specialist and the Indonesia Cancer Foundation, in 2006 CC is the most common cancer in Indonesia. Similarly, data from various academic hospitals in 2007 showed that CC is the most common malignancy in this country, followed by ovary, uterus, vulva and vagina cancers (Aziz, 2009). It is already known that the causative agent of CC is Human Papilloma Virus (HPV). The virus is classified into two groups, *i.e.* high-risk (HR) and low-risk (LR) HP. HR HPV, which sub-divided into HPV genotypes 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 61, 73, and 81, is closely related to the development of CC. In contrast, LR HPV, *i.e.* HPV genotypes 6, 11, 40, 42, 43, 44, 54, 61, 72, and 81, is rarely found in malignant tumors. However, this later HPV is associated with the incidence of warts benign epithelial oral and urogenital in adults and children (Zaravinos et al., 2009; Gutiérrez-Xicotencatl et al., 2009).

Basically, CC can be controlled by applying primary or secondary prevention. As the secondary prevention, Papanicolaou (Pap) smear test is a popular screening tool to determine changes of cells in the cervical area, either in the form of low grade squamous intra epithelial lesions (LSIL) or high grade squamous intra epithelial lesions (HSIL). When suspicious development of cervical malignancy is detected, a proper therapy can be performed soon. For the primary CC prevention, prophylactic HPV vaccination is carried out. There are currently two types of HPV prophylactic vaccines, namely bivalent vaccine for HR HPVs 16 and 18 (Cervarix) and quadrivalent vaccine for LR

and HR HPV 6, 11, 16, and 18 (Gardasil). While the bivalent vaccine can decrease the risk of and cervical dysplasia, quadrivalent vaccine is known to reduce the risk of CC, dysplasia of cervix, vulva, vagina, and genital warts.

HPV vaccination is rather specific; it only provides protection against infection by HPV genotype that is represented in the vaccine. In this respect, the types of HPV applied as a vaccine greatly determines the success of the vaccination. Therefore, to develop an efficacious HPV vaccine that will be used in a particular area, epidemiological HPV genotype data in that area are essential. Unfortunately, in Indonesia, such data are still limited to only several areas, such as in Jakarta, Tasikmalaya, Bali and Bandung, which do not reflect all regions of Indonesia. Thus, here we report genotyping of HPV samples collected from the patients of the Dr. Soetomo Hospital, Surabaya, Indonesia, in 2013.

Materials and Methods

A cross-sectional study was conducted with paraffin block samples of LSIL, HSIL, and SCC cervix that were collected from the patients of the Department of Pathology, Dr. Soetomo Hospital, Surabaya, Indonesia, in the period of January-December 2013. This study was approved by Health Research Ethics Committee of the Faculty of Medicine, Universitas Airlangga (approval No. 298/EC/KEPK/FKUA/2015).

Paraffin blocks were taken randomly for DNA extraction. The preparation processes of the paraffin blocks were conducted as previous study (Siriaunkgul et al., 2008). Paraffin blocks were cut to pieces of ~25 μm^2 (5 µm x 5 µm). Deparaffinization was done by xylol and rehydration with ethanol. DNA was extracted by using Macherey Nagel™ NucleoSpin™ Tissue Kit according to its manual and used as a template for polymerase chain reaction (PCR) experiments. The heating scheme for the PCR experiments was as follows: activation of the DNA-polymerase for 10 min at 95°C, followed by 50 cycles of a 30-sec denaturation at 95°C, a 30-sec annealing at 50°C, and a 30-sec elongation at 72°C. The PCR experiments were ended with a 5-min final step at 72°C. Genotyping of HPV was conducted with Ampliquality HPV-Type Express (AB Analitica) that can detect HPV 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68 (a e b), 69, 70, 71, 72, 73, 81, 82, 83, 84, 87, 89, 90. The type of HPV was reported in the form of band on a blot membrane corresponding to the genotype of HPV.

Results and Discussion

Sample description: This study was performed on 68 paraffin block samples of cervical tissue derived from patients with the age range of 26 to 74 years (Table 1). The patients diagnosed with LSIL or Cervical Intra-epithelial Neoplasia (CIN) 1 were 12 persons, HSIL or CIN 2 and 3 were 27 persons, and SCC were 29 persons. Patients with HSIL were at the levels of CIN 2 (13 patients) or CIN 3 (14 patients). Furthermore, patients with SSC suffered either keratinizing SCC (14 persons) or non-keratinizing SCC (15 persons).

Table 1: Data Characteristics of Patients

Type Lession	Age (Year)	Standart Deviation	Number (Person)
LSIL	29-65	9,49	12
HSIL	26-64	10,06	27
SCC	36-74	9,1	29
Total Patients			68

LSIL=low grade squamous intraepithelial lession, HSIL= high grade squamous intraepithelial lession, SCC= *squamus cell carcinoma*

Table 2: HPV Genotype Distribution of LSIL, HSIL, and SCC Cervix

Type Lession	HPV Genotype	Number (Person)
LSIL	HPV 16	9
	HPV 18	1
	HPV 6, 33	1
	HPV 68,72	1
HSIL	HPV 16	15
	HPV 18	4
	HPV 52	1
	HPV 59	1
	HPV 67	1
	HPV 6, 18	1
	HPV 6, 45	1
	HPV 16, 67	1
	HPV 26, 61	1

	HPV 52, 67	1
SCC	HPV 16	15
	HPV 18	7
	HPV 45	2
	HPV 52	2
	HPV 56	1
	HPV 16, 18	1
	HPV 16, 45	1
Total Patients		68

LSIL=low grade squamous intraepithelial lession, HSIL= high grade squamous intraepithelial lession, SCC= *squamus cell carcinoma*

The genotyping results are presented in Table 2. The results indicated that patients with LSIL are single-infected by HPV 16 or 18, or multiple-infected by HPV 6/33 or 68/72. Similarly, several patients with HSIL are single-infected by HPV 16 or 18. In addition, single HPV infection with other genotypes, *i.e.* 52, 59, and 67, or multiple infections, *i.e.* with HPV 6/33 or 68/72, were identified in samples from HSIL patients. Furthermore, the patients with SCC are commonly single-infected by HPV 16 or 18. However, single infection by HPV 45, 52, or 56, or multiple infections by HPV 16/18 or 16/45, were also found in patients with SCC.

Comparing HPV infection modes found in the samples, the majority of the infection is by a single HPV genotype. It was identified from 86.77% (59/68) samples. On the other hand, infection by multiple HPV genotypes was observed only in 13.23% (9/68) samples. While single infection is shown by HPV genotypes 16, 18, 45, 52, 56, 59, or 67, multiple infection is by a combination of genotypes 6/18, 6/33, 6/45, 16/18, 16/45, 16/67, 26/61, 52/67, or 68/72 (Table 3). Furthermore, as observed in other studies, the most dominant HPV genotype infects patients with cervical precancerous lesions and SCC in this study is HPV 16 (62.68%), followed by HPV 18 (20.9%), HPV 45 (5.97%), 52 (5.97%), and HPV 67 (4.48%) (Table 4).

Table 3: Single-Multiple Infection HPV in Cervical Precancerous Lession and SCC Cervix

Type infection	Number	%
Single infection	59	86,77
HPV 16	39	
HPV 18	12	
HPV 45	2	
HPV 52	3	
HPV 56	1	
HPV 59	1	
HPV 67	1	
Multiple infections	9	13,23
HPV 6, 18	1	
HPV 6, 33	1	
HPV 6, 45	1	
HPV 16, 18	1	
HPV 16, 45	1	
HPV 16, 67	1	
HPV 26, 61	1	
HPV 52, 67	1	
HPV 68, 72	1	
Total Patients 68 persons		100

Table 4: The Prevalence of HPV Genotypes in the Cervical Precancerous Lesions and SCC

HPV Genotype	Number	%
HPV 16	42	62,68
HPV 18	14	20,9
HPV 45	4	5,97
HPV 52	4	5,97
HPV 67	3	4,48

As mentioned above, our data show that HPV infections identified from the patients with LSIL are single infection with HPV 16 or 18, or multiple infections with HPV 6/33 or 68/72. As a comparison, Bao et al. (2007) found HPV genotypes that infect cervical normal in Asia are HPV 16, 18, 31, 33, 35, 39, 51, 52, 56, and 58, and that infect cervical LSIL are 16, 18, 31, 35, 39, 51, 52, 56, 58, and 68. Similarly, a case-control study showed that 26 HPV genotypes were identified in cervical normal and LSIL of Japanese women, *i.e.* HPV 11, 16, 18, 30, 31, 33, 35, 39, 42, 44, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 66, 67, 68, 72, and 73 (Sasagawa et al., 2001). This later study also reported that HPV types 16, 18, 31, 51, 52, and 58 are often associated with the incidence of SCC, and HPV types 16, 31, 33, 35, 45, 51, 52, 56, and 58 are associated with HSIL. Thus, HPV genotypes 16, 18, 31, 51, 52, 58, and possibly HPV 45 and 67 were considered as HR HPV types to Japanese women. HPV types 33 and 55 are associated with HSIL (Sasagawa et al., 2001).

In this study, HPV genotypes that were detected in patients with LSIL are single infections with high-risk HPV genotype 16 or 18, or multiple infections with LR or HR HPV are HPV LR/HR 6/33 and HPV LR/HR 72/68. By increasing their immunity, patient with LSIL have a possibility to heal the infection and remove the virus completely from their body. However, to prevent recurrent disease, it is important to monitor the health of their cervix for ~6 months after healing. On the other hand, it is also a possibility that the LSIL infection develops into HSIL.

HPV genotypes that present in HSIL patients are a single infection with HPV HR 16, 18, 52, 59, 67, and multiple infections with HPV LR/HR 6/18, LR/HR 6/45, HR/HR 16/67, HR/LR 26/61, and HR/HR 52/67. As mentioned earlier, HPV 16, 18, 45, 52, 59, and 67 are classified into HPV HR and HPV 6, 61, 72 are classified as HPV LR

HPV HR types are found in most HSIL and in almost all cervical cancers. Furthermore, the HPV types are also observed in LSIL. It was shown that ~60% HPV infections that lead to LSIL are from high-risk HPV genotypes, but the patient have possibilty to be cure from this infection and the LSIL was not been developing to be HSIL.

HPV types that have been identified on HSIL and SCC in Japan are HPV 16, 18, 31, 33, 35, 45, 51, 52, 56, 58, and 67. In HSIL and SCC, HPV 16 is the most frequent type of infection, followed by HPV 51, 52, 58. The HPV genotype that often infects HSIL in Asia are HPV 16, 58, 52, 18, 33, 51, 31, 56, 35, and 4, as respectively (Boa et al., 2007). On the other hand, HPV types that infects people with HSIL in the world are HPV 16, 31, 33, 58, 18, and in Asia are HPV 16, 58, 52, 18, 51 (Smith et al., 2007).

Genotypes HPV found in the SCC patients are HPV HR 16 (15/29), HPV HR 18 (7/29), HPV HR 45 (2/29), HPV HR 52 (2/29), HPV HR/HR 16/18 (1/29), HPV HR/HR 16/45 (1/29). It is the same with research at Hasan Sadikin Hospital Bandung in patients with SCC that were dominated by HPV 16 (74.2%), 18 (59.1%), 45 (28.8%), 52 (19.7%). Most HPV genotypes in Bandung were HPV 16, 18, 45, 52 (Panigoro et al., 2013; Sahiratmadja et al., 2014; Tobing et al., 2014). HPV often found in Thailand in patients with SCC were HPV 16 (77/96), HPV 52 (10/96), HPV 18 and 33 (both 8/96), and HPV 58 (7/96) (Siriaunkgul et al., 2008). There were 20 types of HPV have been identified on women in Thailand. Five types of high risk HPV mostly found in Thailand were HPV 16, 18, 58, 52, and 45 (Suthipintawong et al., 2011).

The results of this study showed that the prevalence of a single infection on cervical precancerous lesions and SCC was 86.77% (59/68) which HPV 16, HPV 18, HPV 45, HPV 52, HPV 56, HPV 59, and HPV 67. The prevalence of multiple infections was 13.23% (9/68) which HPV 6/18, 6/33, 6/45, 16/18, 16/45, 16/67, 26/61, 52/67, and 68/72. This was in contrast with the results of the study in Bandung that a single infection was 47% and multiple infections were 51.5% (Panigoro et al., 2013). In Thailand there were 75/96 (78.1%) for single infection and 21/96 (21.9%) for multiple infection (Siriaunkgul et al., 2008). The multiple infections appear to be a characteristic in women with cervical abnormalities and were significantly associated with the risk of LSIL, HSIL and SCC. The multiple infections were more common in LSIL compared with malignant lesions (HSIL and SCC) (Sasagawa et al., 2001).

The results of this study indicate that the prevalence of HPV genotypes that infect patients with cervical abnormalities are HPV 16 (62.68%), HPV 18 (20.9%), HPV 45 (5.97%), 52 (5.97 %), and HPV 67 (4.48%). This is similar to the results of research in Bandung Indonesia that the prevalence of HPV that infect the cancer tissue are HPV 16 (90%), HPV 18 (50%), HPV 45 (32.5%), and HPV 52 (30%) (Tobing et al., 2014). HPV types most in Indonesia were HPV 18 (43.0%), 16 (38.0%), 52 (9.1%), 45 (7.4%) and 82 (2.1%) (Smith et al., 2007). The most types of HPV causing cervical cancer are HPV 16 and 18. The results of cervical cancer screening in Jakarta, Tasikmalaya, and Bali Indonesia of 2686 samples were obtained the most common types of HPV that infect the Indonesian women that are HPV 52 (23.2%), HPV 16 (18, 0%), HPV 18 (16.1%) and HPV 39 (11.8%) respectively (Vet et al., 2008). The most often HPV type found in cases of cervical cancer in Jakarta from 74 samples of invasive cervical cancer are HPV 16 (44%), 18 (39%), and 52 (14%) (Schellekens et al., 2004). HPV 16, 18, 45, and 52 is the type that infects most patients with invasive cervical cancer in Bandung (Panigoro et al., 2013; Tobing et al., 2014). This research shown that HPV type that infection patient with cervical precancerous lesions and SCC are HPV 16 (62,68%), HPV 18 (20,9%), HPV 45 (5,97%), 52 (5,97%), and HPV 67 (4,48%).

From our available data, it was difficult to statistically correlate HPV genotypes with their clinical outcomes, *i.e.* LSIL, HSIL, or SCC. However, the data descriptively show that HPV 16 genotype is closely related to clinical outcomes HSIL and SCC, with 38.5% occurrence, while HPV 18 genotype is to SCC, with 58.3% patients.

A Fisher's exact test to examine correlations between single and multiple infections with their clinical outcomes resulted in a significance value of 0.460 ($p > 0.05$). It means that there is no significant correlation between single and multiple infections HPV with their clinical manifestations. Yet, our data show that single HPV infections show a quite high tendency to result SCC (45,8%), while multiple HPV infections give rise to HSIL (55,6%).

Furthermore, correlations between age of patients with HPV clinical outcomes, which were tested by using Spearman's correlation, are quite low. The test gave a significance value of only 0.094 ($p > 0.05$). Although statistically insignificant, there is a tendency that the older patients suffer worse HPV clinical manifestations. The average age of patients with LSIL, HSIL, and SCC are 46.17 (standard deviation 9.485), 46.93 (SD 10.057), and SCC 50.97 (SD 9.658) year, respectively.

After all, HPV genotypes are associated with protection against cervical cancer. Cervical cancer vaccines that is available now day are a bivalent HPV vaccine of the HPV 16/18 and a quadrivalent HPV vaccine of the HPV 6/11/16/18. Vaccination with the bivalent vaccine can provide about 67% cervival cancer protection in Asia. An addition of the genotypes 58, 33, and 52 to the bivalent vaccine resulting a multivalent HPV vaccine that was predicted to increase the protection to about 80%. Furthermore, by involving the HPV genotype 45 in the multivalent vaccine, the protection was expected to be around 87% for Asian people (Boa et al., 2007). In this study, the dominant HPV genotypes that infect patients with cervical precancerous lesions and SCC are HPV that belong to genotypes 16, 18, 45, 52, and 67.

Conclusion

The prevalence of HPV genotype of patient with cervical precancerous lession and SCC from Dr. Soetomo Hospital, Surabaya, Indonesia are HPV 16 (62.68%), then followed by HPV 18 (20.9%), HPV 45 (5.97%), 52 (5.97%), and 67 (4.48%). HPV 16 and 18 have already used as vaccine, and HPV 45 has cross reaction with HPV 18, then HPV 52 and 67 should be considered to develop the second-generation HPV vaccines.

Conflict Interest Statement: The authors declare that there is no conflict of interest.

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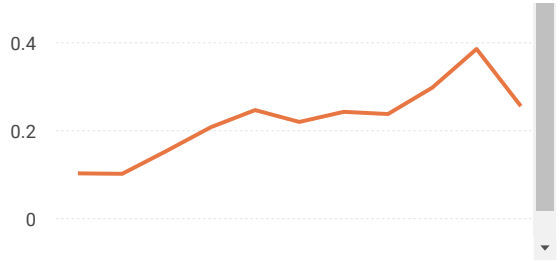

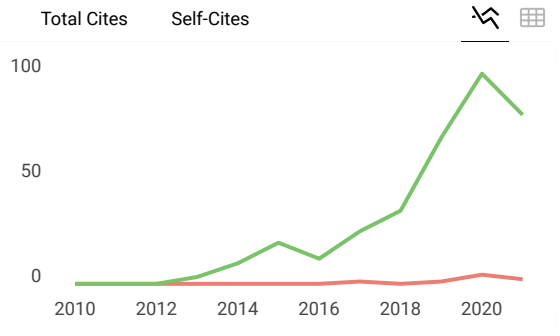

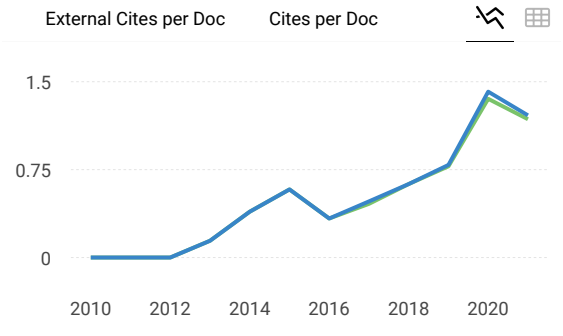

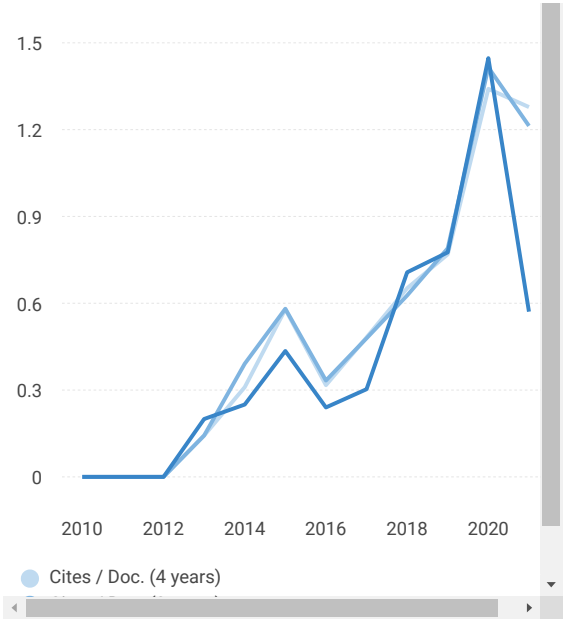

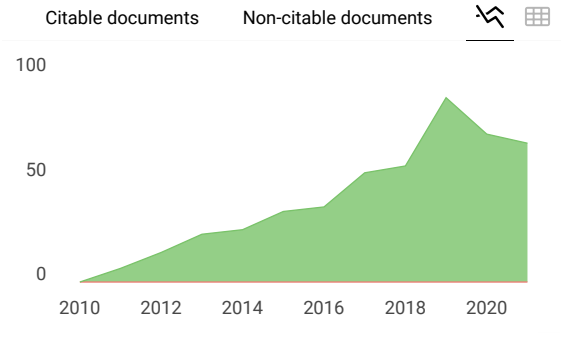

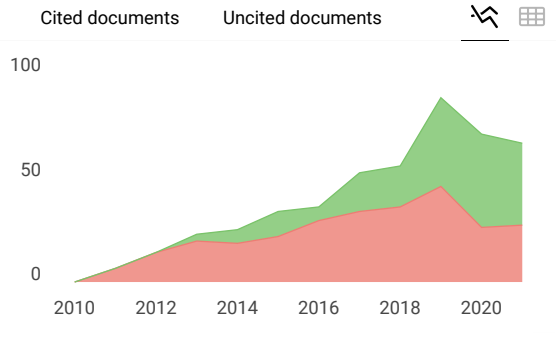

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("ETHICAL CLEARANCE")

No. 298/EC/KEPK/FKUA/2015

KOMITE ETIK PENELITIAN KESEHATAN FAKULTAS KEDOKTERAN UNIVERSITAS AIRLANGGA SURABAYA, TELAH MEMPELAJARI SECARA SEKSAMA RANCANGAN PENELITIAN YANG DIUSULKAN, MAKA DENGAN INI MENYATAKAN BAHWA PENELITIAN BERJUDUL :

EKSPLORASI TIPE HUMAN PAPPILOMA VIRUS YANG MENGINFEKSI PENDERITA SQUAMUS CELL CARCINOMA SEVIKS, ADENOCARCINOMA SERVIKS, DAN KONDILOMA AKUMINATA

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- **Institute of Tropical Disease (ITD) Universitas Airlangga**

DINYATAKAN LAIK ETIK.

Surabaya, 26 Oktober 2015

a.n KETUA KEPK
Wakil Ketua KEPK

Prof. Moersintowarti B. Narendra, dr, MSc, Sp.A(K)