Among Various Epithelial Neoplasms, Epstein-Barr virus involvement is Mainly Restricted to Lymphoepithelial Type of Gastric Carcinoma

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Running title: EBV association with epithelial neoplasm

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Abstract

To demonstrate the association of Epstein-Barr virus (EBV) with primary epithelial neoplasms in the south part of Kyushu, Japan, 761 carcinomas consisting of 75 lung, 61 breast, 107 esophagus, 102 colon, 58 pancreas, 45 thyroid, and 313 gastric cancers were examined by EBER-1 *in situ* hybridization. EBER-1 was detected in 23 cases (7.3%) out of 313 gastric carcinomas, while none of the other carcinomas was positive for EBER-1. Twenty-eight (9.4%) out of 313 gastric carcinomas were poorly to mederately differentiated carcinomas with prominent lymphoid cell infiltration, similar to so-called lymphoepithelioma-like carcinoma, and 19 cases (67.9%) of them were positive for EBER-1. Although two (2.6%) and 11(10.3%) out of 75 lung and 107 esophageal carcinomas were so-called lymphoepithelioma-like carcinomas , respectively, but EBER-1 was not detected in other epithelial neoplasms originating from the lung, esophagus, breast, colon, pancreas and thyroid in the south part of Kyushu, Japan. As a result, EBV was associated with only some gastric carcinomas, but not with other epithelial neoplasms originating from the lung, esophagus, breast, colon, pancreas and thyroid in southern Japan.

Key words: EBV / Epithelial neoplasm / EBER-1 ISH

Introduction

Epstein-Barr virus (EBV) is the causative agent of infectious mononucleosis and is closely related to Burkitt's lymphoma, nasopharyngeal carcinoma, and opportunistic B-cell lymphoma in immunocompromised hosts such as patients with acquired immunodeficiency syndrome (AIDS) or the recipients with organ transplant [Rickinson and Kieff, 1996]. EBV may also be related to the pathogenesis of Hodgkin's disease, peripheral T-cell lymphoma and nasal T/NK-cell lymphoma [Weiss et al., 1987; Harabuchi et al., 1990; Pallesen et al., 1994].

EBV has also been detected in several carcinomas arising in internal organs including the salivary glands, middle ear, sinonasal tract, thymus, esophagus, lung, stomach, breast, biliary tract and uterine cervix [Hamilton-Dutoit et al., 1991; Leung et al., 1998; Leyvraz et. al.,1985; Mori et al., 1994; Butler et al., 1989; Higashiyama et al., 1995; Chan et al., 1995; Shibata et al., 1991; Labrecque et al., 1995; Hsu et al., 1996; Tseng et al., 1997]. Although most of the carcinomas associated with EBV were lymphoepithelioma-like ones, the existence of EBV genome was proven in ordinary carcinomas; that is, the morphological features of EBV-associated carcinomas are not strictly restricted to the lymphoepithelioma-like ones [Tokunaga et al., 1993; Jenkins et al., 1996; Kasai et al., 1994; Wang et al., 1999; Sugawara et al., 1999; Sasagawa et al., 2000]. However, the association of EBV with carcinomas originating in various organs still remains controversial. Even in the lymphoepithelioma-like carcinomas of the lung, EBV association has been reported to depend on ethnic and/or regional background, occurring in Asians but not in Caucasians [Chan et al., 1995].

EBV-encoded small nuclear RNA (EBER) has been shown to be a suitable target for detection of EBV in formalin-fixed, paraffin-embedded tissue sections, because of the high copy number of EBER in latently EBV-infected cells and its stable form

in formalin-fixed, paraffin-embedded sections [Chang et al., 1992]. To demonstrate the regional aspect of EBV association with epithelial neoplasm in the south part of Kyushu, Japan, we examined 761 carcinomas originated from the lung, breast, esophagus, stomach, colon, pancreas and thyroid using highly sensitive EBER-1 *in situ* hybridization (ISH).

Specimens and Methods

Specimens

From 1987 to 1998, 761 patients with primary carcinoma originating in the various organs underwent operations at the First Department of Surgery, Kagoshima University Hospital, Kagoshima, Japan. These included 107 patients with esophageal cancer, 313 with gastric cancer, 75 with lung cancer, 61 with breast cancer, 102 with colon cancer, 58 with pancreas cancer and 45 with thyroid cancer. All the specimens were obtained at surgery, fixed in 10% neutral buffered formalin and embedded in paraffin in a routine manner. After staining with hematoxylin and eosin, the histology of each specimen was carefully reviewed by the pathologists at the Department of Pathology (Table 1).

Preparation of digoxigenin (DIG)-labelled probe

The probe for *in situ* hybridization was 30 mer oligonucleotide complementary to a portion of the EBV-encoded small nuclear RNA-1 (EBER-1). The sequence was 5'-AGACAGCGTCCTCACCACCCGGGACTTGTA-3'. The probe was labelled with DIG-ddUTP at its 3'-end using DIG Oligonucleotide 3'-End Labelling Kit according to the instructions of the manufacturer (Boehringer-Mannheim, Germany).

Pretreatment of tissue sections

The tissue sections (5 μm thick) were fixed on glass slides pretreated with 3-aminopropyltriethoxysilane (Sigma, St. Louis, MO). After deparaffinization by treatment with xyline, the sections were hydrated through graded ethanol series and washed in 2x SSC (1x SSC = 0.15 M sodium chloride + 0.015 M sodium citrate). Next, the sections were refixed in 10% neutral buffered formalin for 20 minutes at room temperature and washed in 2x SSC. After digestion at 37 ℃ for 10 minutes with pronase (1 mg/ml) in 50 mM Tris-HCl (pH 7.5) - 5 mM EDTA, the sections were dipped in glycine (2 mg/ml) in 0.1 M tris-HCl (pH 7,5) - 0.1 M NaCl for two seconds

and washed in 2x SSC.

In situ hybridization

The pretreated sections were reacted at 37 ℃ overnight with the hybridization solution consisting of 500 ng/ml of DIG-labelled antisense probe, 50% formamide, 10% dextran sulfate, 1x Denhart's solution (0.02% Ficoll, 0.02% polyvinyllyrrolidone and 0.02% BSA fraction V), 3x SCC, 100 μg/ml of salmon sperm DNA, 125 μg/ml of yeast t-RNA and 20 mM sodium phosphate buffer (pH 7.4). During the hybridization, the sections were covered with siliconized cover glass and kept in a moist chamber. The EBER-1-positive gastric carcinoma and the sense probe for EBER-1 were used as positive and negative controls, respectively.

Signal detection

After removing cover glass by dipping in 2x SSC, the sections were washed twice in 0.5x SSC for 15 minutes each, and then incubated with 1% skim milk in 5x SCC for 30 minutes. The sections were then reacted with alkaline phosphatase-labeled anti-DIG antibody [diluted to 1:100 in 0.1 M Tris-HCl- 0.15 M NaCl (buffer 1, pH 7.5)] at 37 C for one hour. After washing twice in buffer 1 for 15 minutes each, the sections were dipped twice for 2 minutes each in 0.1M Tris-HCl (pH 9.5)-50 mM $MgCl_2$-4 mM NaCl (substrate buffer). Then the sections were reacted in the dark with 0.04% 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/0.04% 4-nitroblue tetrazolium (NBT) in substrate buffer. When the positive signal was detected in the positive control, the reaction was stopped by washing three times in 10 mM Tris-HCl (pH8.0) -1 mM EDTA for 3 minutes each. After counterstaining with methylgreen for 20 minutes, the sections were washed briefly in distilled water, air-dried, and then mounted with EUKITT (O. Kinder, Germany).

Statistical analysis

Statistical significance was analyzed via chi-square test.

Results

In all 761 primary carcinomas from various organs, EBER-1 was detected in 23 cases (7.3%) out of the 313 gastric carcinomas (Table 1). In these EBER-1-positive 23 cases, EBER-1 signal was localized in the nuclei of almost all tumor cells, but not of adjacent non-neoplastic mucosal cells (Fig. 1A). The prevalence of EBV-positive gastric cancer varied with the location (Table 2). The EBV-positive gastric carcinoma was the most prevalent in the cardia in comparison to those of either the antrum ($p < 0.001$) or the body ($p < 0.05$). In contrast, EBER-1 was not detected in the 75 lung cancers, 61 breast cancers, 107 esophageal cancers, 102 colon cancers, 58 pancreas cancers and 45 thyroid cancers. In the 313 gastric carcinomas, 28 cases (8.9%) were poorly to moderately differentiated ones with prominent lymphoid cell infiltration and classified into so-called lymphoepithelioma-like carcinoma. Nineteen (67.9%) of them were positive for EBER-1(Table 2). Carcinomas with tub2 and por1 showed significantly high prevalence of EBER-1-positive ones in comparison to those with other histological types ($p < 0.01$). Although two (2.7%) and 11 (10.3%) out of the 75 lung and 107 esophageal carcinomas were so-called lymphoepithelioma-like carcinomas, respectively, none of them were positive for EBER-1 (Fig. 1B). A few EBER-1-positive infiltrating lymphoid cells were detected in 6 cases (5.9%), 6 (5.6%), one (2.2%) and 8 (2.8%) of colon, esophagus, thyroid and gastric EBER-1-negative carcinomas, respectively (Fig. 1C).

Discussion

EBV shows tropism for lymphoid and epithelial cells. The association of EBV with gastric carcinomas has been well established over past several years. However, the association of EBV with other epithelial neoplasms has not yet been fully examined. In addition, the association of EBV with some epithelial neoplasms has been reported to depend on ethnic and/or regional background. These lines of evidence prompted us to survey 761 primary carcinomas which originated from seven organs in order to reveal the regional aspect of EBV association with epithelial neoplasms in the south part of Kyushu, Japan.

Primary lymphoepithelioma-like carcinoma of the lung was often associated with EBV in Asians but not in Caucasians [Butler et al., 1989; Gal et al., 1991; Pittaluga et al,1993; Higashiyama et al., 1995; Chan et al., 1995]. Kasai et al. [1994] reported that 5 cases (6.2%) of the 81 lung cancers in northern Japan were associated with EBV and indicated that the morphological features of EBV-associated lung cancers were not restricted to the lymphoepithelioma-like carcinoma. However, Chan et al. [1995] detected EBV in seven lymphoepithelioma-like carcinomas, but not in Chinese 59 lung carcinomas with other histological types. In the south part of Kyushu, Japan, none of the 75 lung carcinomas, including two lymphoepithelioma-like ones, were positive for EBV.

Lespagnard et al. [1994] failed to detect EBV in 10 medullary carcinomas of the breast in Belgium. However, Labrecque et al. [1995] reported that 19 cases (21%) of the 91 breast carcinomas in the United Kingdom were positive for EBV by PCR. They also found that 12 cases (63%) and six cases (31.5%) of PCR-positive samples were also positive for EBV DNA and RNA by ISH , respectively. In their study, no significant correlation was found between the presence of EBV and the histological type of the breast tumor. On the contrary, Chu et al. [1998] and Glaser

et al. [1998] failed to demonstrate the EBV association with 60 cases and 125 cases of the breast carcinoma in Taiwan and in the United States, respectively. We also could not detect EBV in the 61 breast carcinomas in the south part of Kyushu, Japan.

Mori et al. [1994] reported the first case of EBV-associated esophageal carcinoma with lymphoepithelioma-like histology. After that, Jenkins et al. [1996] and Wang et al. [1999] detected EBV in 5 of 60 cases (8.3%) in Europe and 11 of 31 cases (35.5%) in Taiwan, respectively. In the former study, however, EBV-positive 5 cases were not confirmed by ISH. In the latter study, seven out of 11 EBV-positive tumor cases were also EBV-positive at various sites of the nontumor esophageal mucosa. In contrast, Lam et al. [1995] and Wang et al. [1999] did not find the EBV association with 74 cases in Hong Kong and 51 cases in the high-risk area of north China, respectively. In the south part of Kyushu, Japan, none of the 107 esophageal carcinomas, including 11 lymphoepithelioma-like ones, were positive for EBV.

Tokunaga et al. [1993] reported that 66 cases (6.9%) of the 999 gastric carcinomas collected from 1976 to 1992 in southern Japan was positive for EBER-1 and that EBV-positive carcinoma was predominant in the middle to the cardia of the stomach. Recently, Takano et al. [1999] found that 33 cases (6.4%) of the 513 gastric cancers collected from 1994 to 1995 in central Japan was associated with EBV, but could not find the predominance of the EBV-positive cancer location in the body and the cardia of the stomach. The difference of the predominant location of EBV-positive gastric cancer between central and southern Japan may be either due to the collection period or due to regional background. We analyzed the 313 gastric carcinomas collected from 1987 to 1998 in the south part of Kyushu, Japan, resulting in the almost same prevalence and predominance of the location of EBV-positive carcinomas as Tokunaga et al. [1993] did. As a result, the difference in

predominant location may be due to regional background. To confirm this, however, more comparable studies should be conducted in various regions.

None of the 102 colon cancers in the south part of Kyushu, Japan, were positive for EBV. This result was concordant with one case with lymphoepithelioma-like histology in Japan and 36 cases of the colorectal carcinomas in Hong Kong [Vilor and Tsutumi, 1995; Yuen et al., 1994]. None of the thyroid and pancreas carcinomas were also positive for EBV.

EBV was associated with only some gastric carcinomas. Yoshiyama et al. [1997] reported that cell-free EBV could not infect any epithelial cell line but that the epitheplial cell lines were infected *in vitro* with EBV by co-culture of them with EBV-infected B cells. They also showed that the efficacy of EBV infection varied with individual epithelial cell lines. Although the majority of infiltrating lymphocytes were reactive T cells [Uemura et al., 1994], a very few EBER-1-positive B cell may transmit EBV to some type of epithelial cells.

In conclusion, EBV involvement seems to mainly restrict to lymphoepithelial type of gastric carcinoma in the south part of Kyushu, Japan.

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Legend to Figure

Figure 1. EBER-1 *in situ* hybridization in carcinoma sections.

(A) The serial sections of gastric carcinoma were perfomed HE staining (upper) and EBER-1 *in situ* hybridazation (lower). Intensive nuclear hybridization signal was shown in almost all the tumor cells, but not in nontumor cells. Original magnification was x40. (B) In the so-called lymphoepithelioma-like esophageal carcinoma section, no hybridization signal in both tumor cells (arrowhead) and infiltrating lymphoid cells (arrow). Original magnification was x400.

(C) A few infiltrating lymphoid cells (arrow) into esophageal carcinoma were positive for EBER-1. Original magnification was x400.

Table 1. Results of EBER-1 *in situ* hybridization in parafffin sections of epithelial neoplasms from seven organs in the south part of Kyushu, Japan

Organ	Histological type	No. of positive / Total no. of cases	LE-like carcinoma[a] pos. no. / total no.
Lung	squamous cell carcinoma	0/31	0/1
	adenocarcinoma	0/34	
	small cell carcinoma	0/3	
	large cell carcinoma	0/3	0/1
	adenoid cystic carcinoma	0/1	
	adeno-squamous carcinoma	0/3	
Breast	adenocarcinoma	0/61	
Esophagus	squamous cell carcinoma	0/106	0/11
	adenocarcinoma	0/1	
Colon	adenocarcinoma	0/102	
Pancreas	adenocarcinoma	0/58	
Thyroid	papillary carcinoma	0/45	
Stomach	adenocarcinoma	23/313 (7.3%)	19/28 (67.9%)

a) Poorly to moderately differentiated carcinoma with a prominent lymphoid cell infiltration

Table 2. Clinicopathological findings of EBER-positive and -negative gastric carcinomas

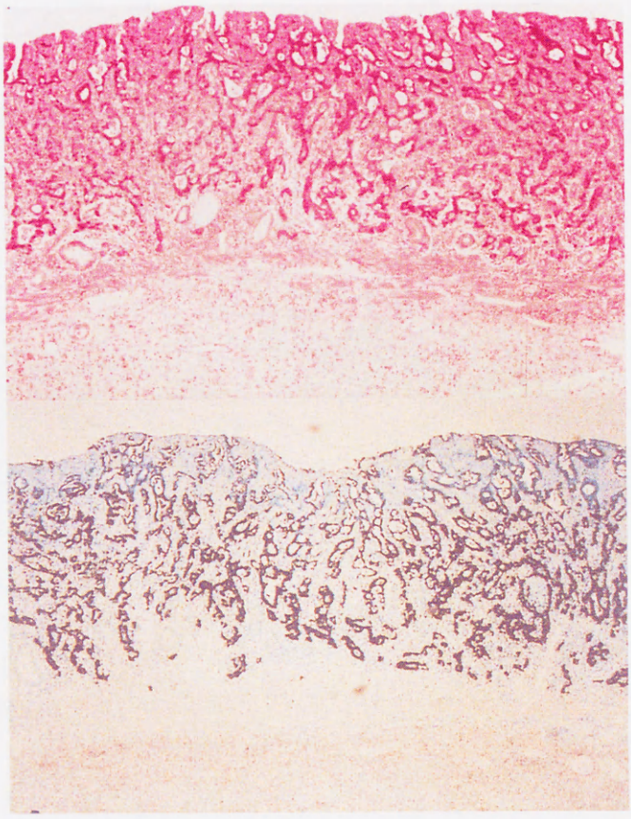
Characteristics	EBER-positive (n=23)	EBER-negative (n=290)	*P*-value[c]
Age:	62.65±10.2	64.53±11.5	
Sex:			
Male	17	199	p=0.62
Female	6	91	
Location:			
Antrum	1	105	* p<0.05
Body	8	75	
Cardia	14	110	** p<0.01
Depth of invasion:[a]			
t1	11	117	
t2	7	108	
t3	4	53	
t4	1	12	
Histological type:[b]			
pap	0	6	
tub1	1	82	** p<0.01
tub2	9	80	
por1	8	16	
	6 17 (73.9%)	194 96 (33.1%)	
por2	3	69	
sig	1	28	
muc	0	8	
ud	1	1	
Lymph node invasion:			
(+)	8	125	p=0.45
(-)	15	165	

a) t1:mucosa , submucosa; t2:muscularis propria, subserosa; t3:serosa exposed; t4:serosa infiltrating

b) pap:papillary adenocarcinoma; tub1:well differentiated adenocarcinoma; tub2:moderately differentiated adenocarcinoma; por1:soild type, poorly differentiated adenocarcinoma; por2:non-solid type, poorly differentiated adenocarcinoma; sig:signet-ring cell carcinoma; muc:mucinous carcinoma; ud:undifferentiated adenocarcinoma

c) Statistical significance was evaluated via chi-square test.

Figure 1



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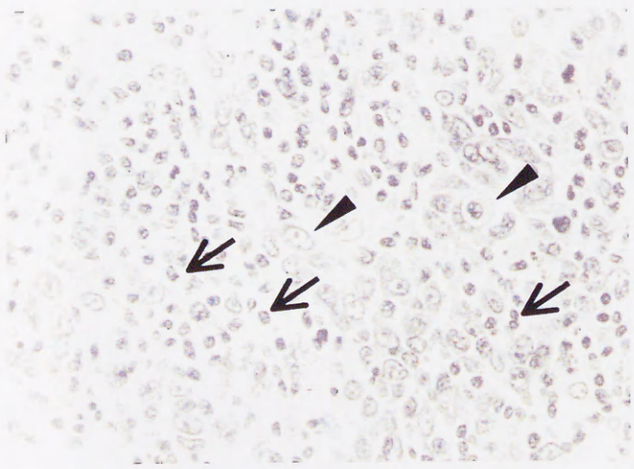

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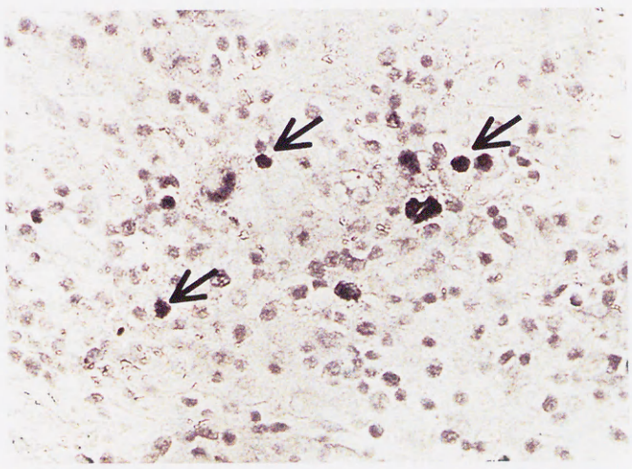

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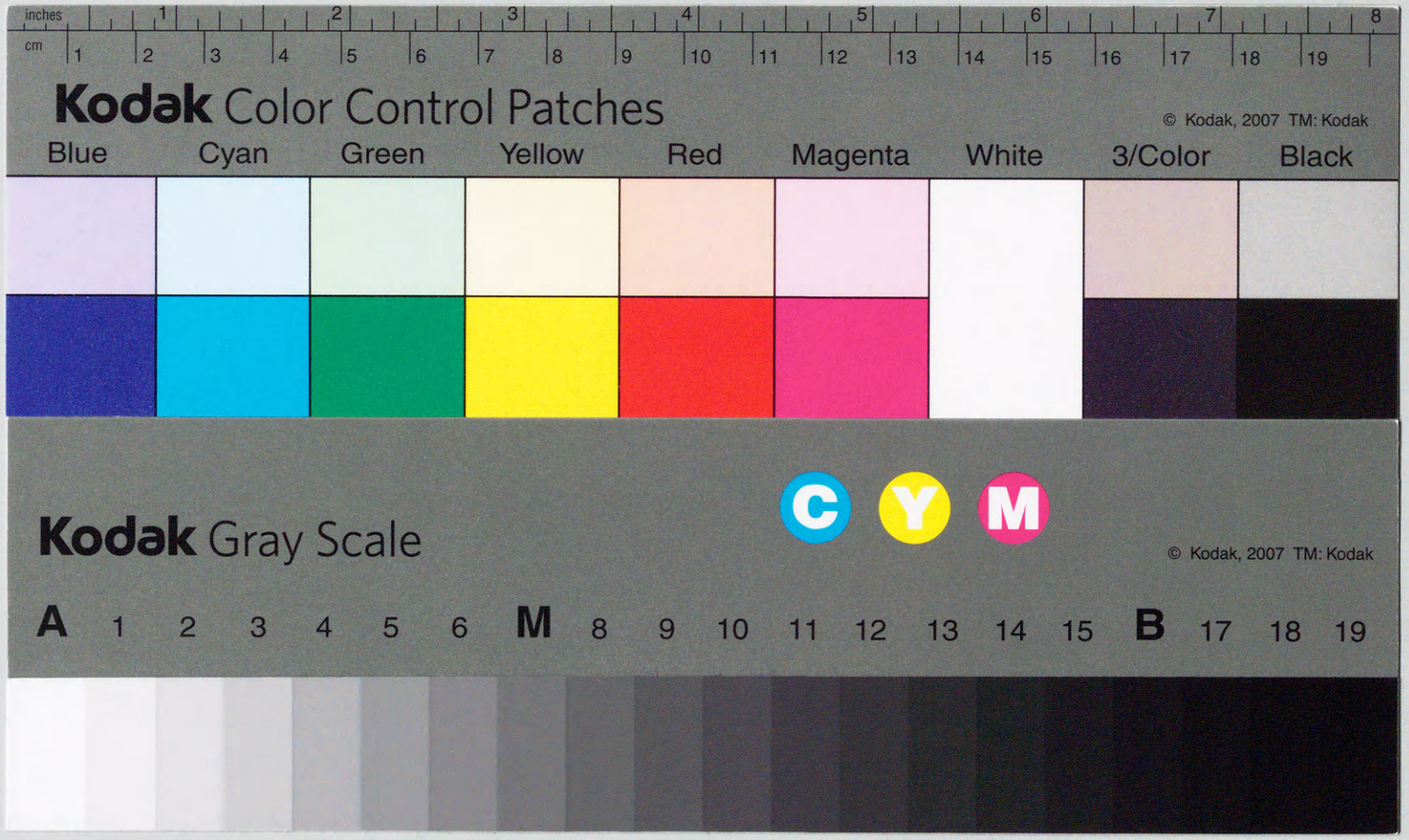
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