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Apoptosis and cellular proliferation in oesophageal squamous cell carcinomas: differences between keratinizing and nonkeratinizing types

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Abstract To assess cell death and cellular proliferation activity, the apoptosis index, the Ki67 proliferative index and overexpression of p53 protein were evaluated in 69 oesophageal squamous cell carcinomas (ESCC), all surgically resected from Japanese patients. Apoptosis was examined by Gavrieli's method in histological sections, and proved to be significantly related to keratinization and ESCC progression. Overall labelling indices were 15.68±4.04 (positive/1,000 nuclei) and 6.79±0.64 respectively, in keratinizing and nonkeratinizing types. The apoptosis labelling index increased, especially in keratinizing lesions, from 4.50±0.59 with cancer invasion to mucosa through 11.46±2.70 with involvement of the submucosa up to 21.18±3.72 in cases of penetration to the muscularis propria or adventitia. The relationship between apoptosis, Ki67 scores and p53 expression was determined in identical cancer nests on serial sections. An inverse correlation was shown between the apoptosis score and the Ki67 score in both keratinizing and nonkeratinizing types. There was no significant correlation between apoptosis score and p53 expression, either overall or separately in keratinizing or nonkeratinizing types of ESCC. Our results suggest that a mechanism of induction of apoptosis similar to that operating in normal epidermis acts in keratinizing ESCC, and that as tumour volume increases, single cell death becomes more frequent.

Key words Oesophageal squamous cell carcinoma · Apoptosis · Cell growth · p53 Protein

Introduction

Cancer research has traditionally been directed toward cell proliferation. However, apoptosis has increasingly been attracting oncologists' attention [12]. While it is found in virtually all untreated malignant tumours [21], there have been only few reports [6, 18] of the occurrence of apoptosis in squamous cell carcinomas. In these, apoptosis was identified on the basis of light and electron microscopic criteria [11].

Gavrieli et al. [7] recently published a method for the in situ visualization of apoptosis via specific labelling of nuclear DNA fragmentation. Gavrieli's method is based on the specific binding of terminal deoxynucleotidyl transferase(TdT) to 3'-OH ends of DNA breaks, ensuing synthesis of a polydeoxynucleotide polymer. After exposure of nuclear DNA in sections by proteolytic treatment, TdT is used to incorporate biotinylated deoxyuridine at sites of DNA breaks. The signal can be amplified by avidin-peroxidase, enabling conventional histochemical identification at the light microscope level. Thus, Gavrieli's method allows in situ visualization of apoptosis at the single cell level in formalin-fixed, paraffin-embedded tissue sections.

In the present study, we used this method to detect apoptosis in oesophageal squamous cell carcinomas, concentrating attention on its association with differentiation, cancer progression, and cell proliferation rate. Squamous cell carcinoma frequently shows keratinization, which is not found in other malignant tumours. We were concerned with the relationship between apoptosis and keratinization, and therefore compared the occurrence of apoptosis in two groups comprising keratinizing and nonkeratinizing squamous cell carcinomas. Much of the current interest in apoptosis stems from the discovery that it can be regulated by certain proto-oncogenes and the *p53* tumour suppressor gene [13]. In the present study, we also investigated whether the *p53* gene product enhances or suppresses apoptosis induction in oesophageal squamous cell carcinoma.

Materials and methods

Materials

Surgically removed materials from 69 Kitasato University Hospital patients with oesophageal squamous cell carcinoma (ESCC)

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were used. Formalin fixation was used before paraffin embedding. Of the 69 tumours, 20 were well differentiated (WD), 11 moderately differentiated (MD), and 38 poorly differentiated (PD). ESCCs were divided into keratinizing type (KT, 31 cases) and nonkeratinizing type (NKT, 38 cases), the former including WD and MD tumours, and the NKT only the PD tumours. The depth of tumour invasion was classified as mucosa (m, 10 cases), submucosa (sm, 30 cases), and muscularis propria or adventitia (mp-a, 29 cases).

Tissue preparation

Tissue samples were fixed in 10% formalin and embedded in paraffin. Paraffin sections 4μm thick on silane-coated slides were placed in an oven at 37°C overnight.

DNA nick end labelling of tissue sections

We followed Gavrieli's method (TUNEL: TdT-mediated dUTP-biotin nick end labelling) [7] in performing this procedure. Proteins were stripped from nuclei of tissue sections by incubation with 100 μg/ml proteinase K for 15 min at room temperature. TdT(0.3 e.u./μl) and biotinized dUTP in TdT buffer were then added to the sections before incubated in a humid atmosphere at 37°C for 60 min. The conventional avidin-biotin complex method was carried out subsequently, and diaminobenzidine was used for the coloration.

Immunohistochemistry for Ki67 and p53

Serial paraffin sections were obtained from the same blocks as were used for TUNEL staining. The sections were heated in 0.01 M citrate buffer in the microwave oven for 15 min. Routine immunohistochemistry was carried out with the avidin-biotin-peroxidase method. The specific antibodies used were as follows; a rabbit anti-human Ki67 antigen (Dako, Copenhagen, Denmark, dilution 1:150), and a rabbit anti-human p53 protein (CM1 from Novocastra Laboratories, Newcastle, UK, 1:800). p53 Immunoreactivity pattern was determined in serial sections for the cancer nests corresponding to those from which ApoLI values were calculated. Overexpression of p53 was evaluated as follows: (1) diffuse positivity; (2) peripheral positivity, in which cancer nuclei in the peripheral zone of the cancer nest are immunostained; (3) focal positivity with groups of immunostained cancer cells; and (4) scattered positivity, with sparse cancer cells showing clear-cut immunoreactivity. Scattered positivity and lack of p53 expression were classified as p53(-).

Counting procedure for assessment of ApoLI and Ki67

Five cancer nests were arbitrarily chosen from keratinizing nests with apoptotic cells in KT ESCC, and from nonkeratinizing nests with apoptotic cells in NKT ESCC. Cancer cells and TUNEL-stained nuclei were counted in these five nests (corresponding to a total of 1400–5200 cancer cells) per case. The apoptosis labelling index (ApoLI) per case was expressed per mille, that is to say as the mean number of TUNEL-stained nuclei per 1000 cancer cells. The Ki67 labelling index (KiLI) was determined in serial sections for the cancer nests corresponding to those from which ApoLI values were calculated. Cancer cells and Ki67-positive cancer cells were counted in these five nests, and KiLI was also expressed per mille, (mean number of Ki67-positive cells per 1000 cells).

Statistical analysis

ApoLI and KiLI are presented as mean values ± standard error (SE). They were compared with the grade of cancer cell differentiation and cancer progression, and between keratinizing and nonkeratinizing squamous cell carcinomas, by means of the Mann-Whitney U-test.

Results

Apoptosis

Normal squamous epithelium distant from squamous cell carcinomas was examined in ESCC tissue sections of 20 cases (Fig. 1). The background ApoLI was 4.27±0.58 (mean±SE). ApoLIs of ESCC overall and for WD, MD and PD were 10.92±1.30, 18.50±3.38, 11.40±3.39 and 6.79±0.64, respectively (Table 1, Figs. 2, 3). ApoLIs of KT sm, mp-a carcinomas and NKT mp-a carcinomas were significantly enhanced compared with that of nor-

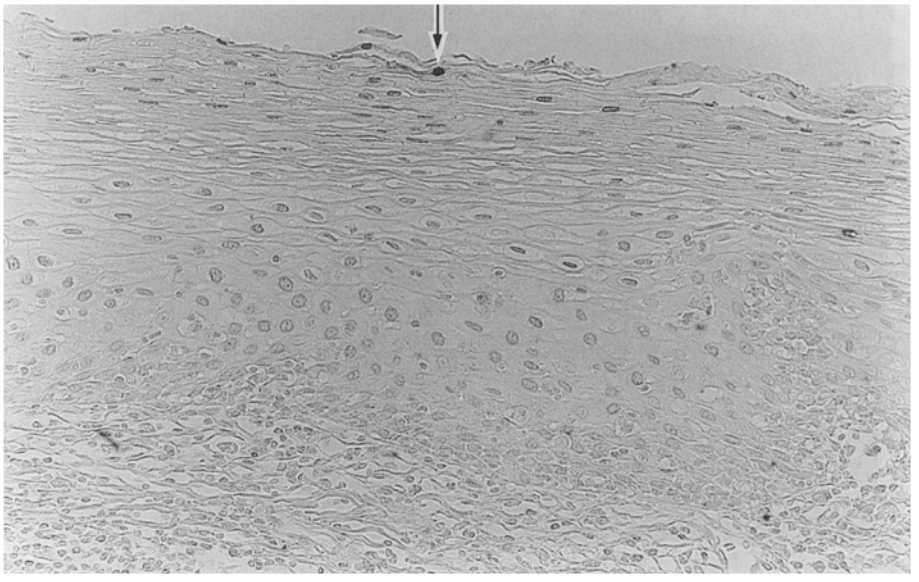

Fig. 1 A superficial cell (*arrow*) stained by the TUNEL method in normal squamous epithelium. ×250

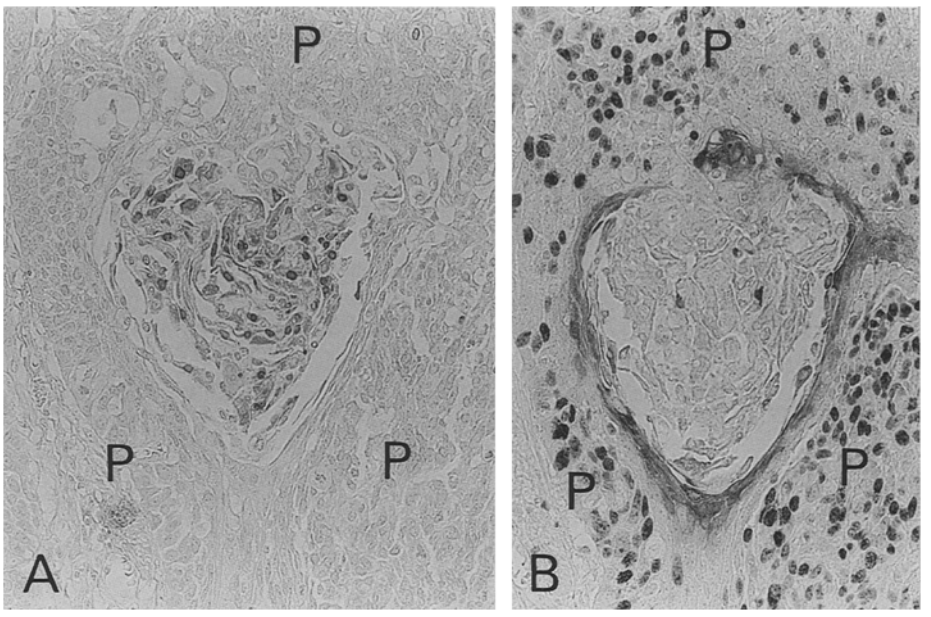

Fig. 2 **A** Detection of apoptosis by TUNEL staining. TUNEL-stained cancer cells are more frequently found in the central zone than in the peripheral zone of a keratinizing cancer nest. ×250. **B** Immunohistochemical detection of Ki67 antigen. Ki67-positive cells are much more frequently observed in the peripheral zone (*P*) than in the central area of the keratinizing cancer nest shown in **A**. ×250

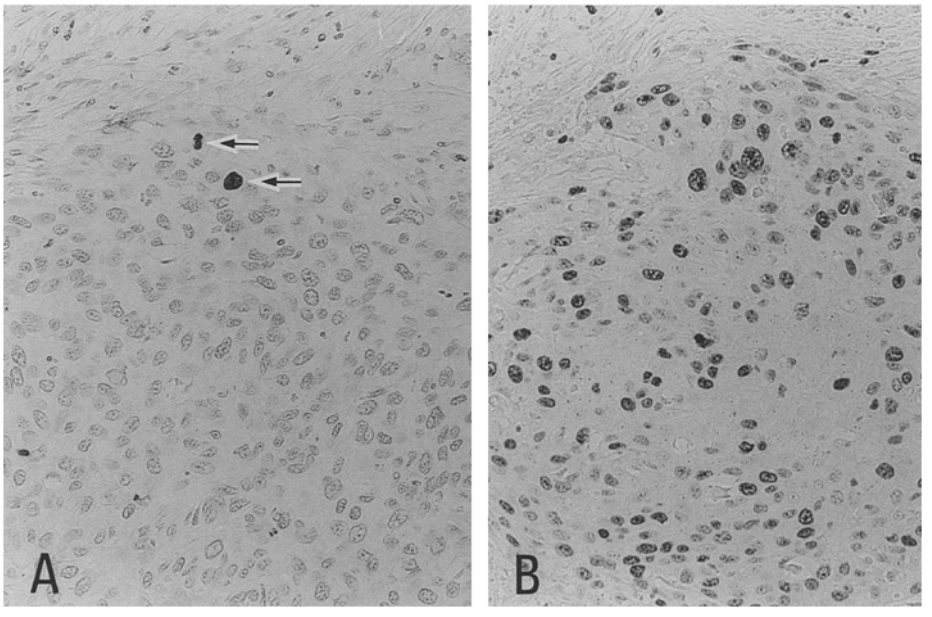

Fig. 3 **A** TUNEL-stained cells (*arrows*) are scattered in a nonkeratinizing cancer nest. ×250. **B** Most cancer cells are positive for Ki67 in the nonkeratinizing cancer nest shown in **A**. ×250

mal squamous epithelium($P<0.01$, $P<0.0001$, $P<0.01$, respectively, Tables 1,2). ApoLI was also significantly elevated in differentiated cancers (WD vs PD; $P<0.001$), and significantly higher in KT than in NKT (overall cases; $P<0.001$, sm; $P<0.01$, mp-a; $P<0.05$, Table 2).

As cancers progressed, overall ApoLI became higher (m vs mp-a; $P<0.001$, sm vs mp-a; $P<0.001$, Table 2). In KT, the sm value was significantly higher than that for m carcinomas ($P<0.01$). ApoLI of mp-a carcinomas was significantly higher than those for m and sm carcinomas (respectively $P<0.01$, $P<0.05$). In NKT, there was no significant difference in ApoLI between m and sm, or between m and mp-a cases. However, a significant difference was found between sm and mp-a cases ($P<0.05$). Observation of ApoLI at each depth, including mucosal, submucosal layers and muscularis propria or adventitia, did not reveal consistent differences in sm and mp-a carcinoma cases. (These data are not shown.)

Comparison of ApoLI and Ki67 immunoreactivity

The KiLI of ESCC was significantly enhanced in comparison with that of normal squamous epithelium, with the PD value being significantly higher than that for WD lesions ($P<0.001$, Table 1) and the NKT KiLI being significantly higher than that of KT ($P<0.01$, Table 2, Figs. 2, 3). No significant correlation of Ki67 immunoreactivi-

Table 1 Apoptosis and Ki-67 labelling indices in oesophageal cancer cases (*ApoLI* apoptosis labelling index, *ESCC* oesophageal squamous cell carcinoma, *KiLI* Ki67 labelling index, *MD* moderately differentiated, *PD* poorly differentiated, *WD* well differentiated)

	Normal mucosa (n=20)	ESCC (n=69)			
		WD (20)	MD (11)	PD (38)	Total
ApoLI	4.27±0.58	18.50±3.38[a]	11.40±3.39[b]	6.79±0.64[c, d]	10.92±1.30[e]
KiLI	84.59±13.05	248.33±79.25[a]	375.21±23.67[e]	371.49±21.10[a, d]	335.70±19.28[a]

[a–e] Significant differences; [a] $P<0.0001$, [b] $P<0.01$, [c] $P<0.05$, [e] $P<0.001$ for difference from value for normal mucosa; [d] $P<0.001$ for difference from value for WD

Table 2 Apoptosis and Ki67 labelling indices in keratinizing and nonkeratinizing oesophageal cancers (*KT* keratinizing ESCC, *NKT* nonkeratinizing ESCC, *m* ESCC invading mucosa, *sm* ESCC invading submucosa, *mp-a* ESCC invading muscularis propria or adventitia)

	KT (n=31) Mean±SE	NKT (n=38) Mean±SE	Total (n=69) Mean±SE
ApoLI			
m	4.50±0.59 (5)	5.84±1.06 (5)	5.17±0.61 (10)
sm	11.46±2.70* (8)	5.93±0.82[a] (22)	7.41±1.01 (30)
mp-a	21.18±3.72*, † (18)	8.95±1.28[b, †] (11)	16.54±2.59**, ‡ (29)
Total	15.68±4.04	6.79±0.64[c]	10.92±1.30
KiLI			
m	292.56±55.96	380.76±67.03	341.57±44.83
sm	318.56±75.79	352.12±19.45	342.86±24.49
mp-a	275.20±43.28	416.55±63.55	324.37±37.82
Total	291.56±26.42	371.49±21.10[a]	335.70±19.28

[a, b, c] Significant difference from the value for KT: [a] $P<0.01$, [b] $P<0.05$, [c] $P<0.001$

*, ** Significant difference from the value for m: * $P<0.01$, ** $P<0.001$

†, ‡ Significant difference from the value for sm: † $P<0.05$, ‡ $P<0.001$

Table 3 Relationships between apoptosis and Ki67 labelling indices and expression of p53

	p53 (–)[a] (n=24)	p53 (+) (n=45)			
		Diffuse pattern (13)	Peripheral pattern (17)	Focal pattern (15)	Total
	Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE
ApoLI					
KT	16.06±4.94 (13)	9.69±2.75 (8)	12.12±2.63 (15)	11.99±3.39 (11)	11.50±1.68 (34)
NKT	6.97±1.10 (11)	15.06±10.27 (5)	12.13±3.59 (2)	7.75±1.34 (4)	6.75±1.21 (11)
Total	12.01±2.83	11.76±4.10	12.12±2.33	10.86±2.53	10.34±1.33
Ki67					
KT	268.07±29.55	394.79±57.75	360.25±42.19	334.87±57.84	361.90±28.89*
NKT	384.21±52.64	326.35±112.00	451.89	246.30±65.84	338.80±56.01
Total	299.74±27.59	368.47±53.83	367.30±39.45	307.62±44.91	355.98±25.51

* Significant difference from the value for p53 (–): $P<0.05$

[a] p53 (–) comprises scattered positivity and lack of p53 expression cases

ty with cancer progression was revealed. Significant differences of KiLI were not observed among tumour localizations (mucosal, submucosal and muscularis propria or adventitia) in sm and mp-a carcinoma cases. (These data are also not shown.)

KiLI showed no overall linear correlation with ApoLI (r=0.09, P=0.486). There was also no significant linear correlation between KiLI and ApoLI in KT, NKT, m, sm, and mp-a cases. However , an inverse correlation was shown between ApoLI and KiLI in KT and NKT with ApoLI being significantly higher, while conversely, KiLI was significantly lower in KT than in NKT (Table 2).

Comparison of apoptosis and overexpression of p53

p53 immunoreactivity was restricted to tumour cell nuclei of 45/69 ESCC (65%). The predominant overexpres-

sion pattern was diffuse positivity in 13 cases (19%), peripheral in 17 cases (24%) and focal in 15 cases (22%). ApoLI values of p53-positive and p53-negative tumours were 10.34±1.33 and 12.01±2.83, respectively, with no significant difference (Table 3). There was also no significant correlation between p53-positive or negative staining and tumour type (KT or NKT). When dividing p53 immunoreactivity into four groups of p53-negative, diffuse, peripheral and focal, no overall significant difference was found among these groups or for KT and NKT cases. The KiLI was significantly higher in p53-positive tumours than in p53-negative ones only in the KT cases ($P<0.05$).

Discussion

The present results, obtained with the TUNEL method, show that apoptosis is significantly more frequent in KT than in NKT, as well as increasing gradually with the progression of cancers (Table 2). There have been few reports describing any association between apoptosis and keratinization. McCall and Cohen [16] demonstrated that DNA fragmentation was initiated in the granular keratinocyte layer of newborn BALB/c mouse skin, whereas DNA extracted from basal keratinocytes was intact. They suggested that basal keratinocytes, in preparing for terminal differentiation, DNA fragmentation, and death, would synthesize "death proteins", including endonuclease. In addition, the same basal keratinocytes would synthesize inhibitors of these death proteins, so that the programme would not be prematurely expressed. They further postulated that the half-life of the inhibitor was shorter than that of the death proteins. If inhibitor synthesis decreases as the basal cell moves upwards, the death programme would automatically triggered. TUNEL staining in the present case showed apoptosis to increase as cancer cells became located more centrally in keratinizing cancer nests. These results suggest that the same mechanism of apoptosis induction as in normal epidermis may act in keratinizing ESCC.

Ki67 is a human nuclear antigen expressed by all proliferating cells [8] during late G1, S, M and G2 phases of the cell cycle, while cells in G0 phase consistently lack the antigen [9]. A significantly higher incidence of Ki67 expression in PD than in WD (Table 1), and in NKT than in KT, was found in the present study (Table 2). Since the apoptosis score was significantly higher in KT than in NKT, these results show an inverse correlation between apoptosis and Ki67 when KT and NKT types of ESCC are considered separately.

Leoncini et al. [14] reported a significant positive correlation between the apoptotic indices and the proliferative indices (percentage of Ki67 positive neoplastic cells) in malignant non-Hodgkin's lymphoma. The discrepancy between their findings and our results might have arisen because keratinization, which is observed exclusively in squamous cell carcinomas, plays an important part in apoptotic induction and the decrease in Ki67 positivity in ESCCs. Our findings demonstrate that ApoLIs increase and KiLIs decrease as keratinization starts in cancer nests. Thus, KT ESCCs show high ApoLIs and low KiLIs. Keratinization is much less pronounced in NKT than in KT ESCCs, so that ApoLIs decrease and conversely KiLIs are enhanced. These findings might explain an inverse correlation between ApoLI and KiLI in ESCCs.

In adenocarcinomas of other organs, apoptosis is generally more common in poorly differentiated than in well-differentiated lesions. For example, Aihara et al. showed significantly larger numbers of apoptotic bodies in poorly differentiated carcinomas than in well-differentiated prostate carcinomas [1]. The difference in ApoLIs between ESCC and adenocarcinomas in terms of association with differentiation can also explained by the keratinization.

ApoLI increased as cancer progressed more extensively in ESCC, especially in KT (Table 2). In other words, apoptosis induction is more frequent in the advanced stages of this type of cancer, indicating the involvement of factors other than keratinization. Mild ischaemia [12], tumour necrosis factor-α released by infiltrating macrophages [2, 20] and cytotoxic T-lymphocytes [3] are generally known to play positive parts in inducing apoptosis in tumours. The influence exerted by these factors may thus be particularly pronounced in advanced stages of ESCC.

It is now widely accepted that *p53* gene alterations are the most common changes that occur during malignant progression of diverse types of human cancer [10, 15]. Cancer cells with alterations in this gene have frequently been found to express readily detectable levels of p53 protein, and thus such overexpression is considered to be a valid indirect parameter for estimating the percentage of ESCC in which *p53* gene mutations have occurred [4]. Wild-type *p53* immunoreactivity is not usually detectable in normal tissues, owing to its very short half-life [17].

Wagata et al. [19] reported that *p53* mutations were observed in 5 of 5 p53(+++) cases (almost all cancer cells stained), 2 of 2 p53(++) cases (more than 50% of cells stained), 3 of 6 p53(+) cases (under 50% of cells stained), 4 of 16 p53(-) cases (most cells not stained). Hence, scattered positivity, as well as lack of p53 expression, was classified as p53(-), because scattered positivity means most cancer cells were not stained.

A major mechanism whereby abnormalities of *p53* contribute to the development and progression of tumours may be abrogation of the normal pathway that leads to the self-destruction of mutant cells [13]. Eliyahu et al. [5] also estimate that p53 mutant proteins may plausibly contribute to carcinomatous progression both by inactivating endogenous wild-type *p53* and by triggering oncogenes. The ESCC series examined by us showed a high rate of p53 overexpression (65%), but no correlation was demonstrated between apoptosis score and p53 expression, either overall or in KT and NKT examined separately (Table 3). Furthermore, the expres-

sion pattern of p53 was also not associated in any way with the apoptosis score so far as examined immunohistochemically. There is a limitation of immunohistochemical methodology for precise investigation of the relationship between apoptosis and p53, because it is necessary to examine the correlation at the single cell level.

In conclusion, the present study findings clearly document a significant difference in ApoLI between KT and NKT types of ESCC, and demonstrate a positive correlation between ApoLI and cancer progression. KiLI was lower in KT than NKT, while the situation with ApoLI was the reverse.

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