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The importance of FISH signal cut-off values for 9p21 deletion in malignant pleural mesothelioma: Is it underestimated?

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ABSTRACT

Malignant mesothelioma (MM) is an aggressive cancer with a poor prognosis. The most common genetic alteration in MM is the deletion of the INK4a/ARF locus, which encodes the p16 protein and is located on the short arm of chromosome 9 (9p21). Recently, it has been shown that homozygous deletion of 9p21 has both diagnostic and prognostic significance in MM. It is a known fact that, to interpret fluorescence in situ hybridization (FISH) signals, a cut-off value for each probe should be determined for a correct diagnosis. To our knowledge, there is no consensus or confirmed protocol for cut-off values to evaluate FISH signals in MMs. Therefore, the aim of our research was to address 9p21 deletion status and p16 expression profiles of MM by determining our own cut-off values and the effectiveness of using p16 negativity and 9p21 deletion as markers for differentiating MMs from benign mesothelial proliferations in 114 cases. We established a cut-off value for the detection of 9p21 deletion by using 13 benign reactive cases (6 reactive mesothelial hyperplasias and 7 chronic fibrinous pleuritis cases) and found between 0–7%. According to our calculations, homozygous deletion was defined by loss of both p16 gene signals in at least 13.3% of the nuclei that showed at least 1 signal for the CEP 9 probe. Our FISH results showed homozygous 9p21 deletion in 82 of the 114 cases of MM (71.9%), and p16 expression was negative in 75 of the 114 cases (65.8%). The correlation between loss of p16 protein expression and 9p21 deletion was statistically significant. Among the p16-negative cases, 86.7% also had the 9p21 deletion.

The combined examination of the 9p21 deletion and loss of p16 expression is helpful for diagnostic purposes, but because the FISH method is an expensive technique and loss of p16 expression is not specific for mesotheliomas, p16 negativity can guide practitioners to eliminate cases that require further investigation by FISH. The variability in the significance of 9p21 homozygous deletion results from inconsistencies among different institutes, suggesting that each institute should establish its own cut-off value using reactive mesothelial proliferations. Alternatively, global studies are needed to assess cut-off values.

1. Introduction

Malignant mesothelioma (MM) is an aggressive cancer that originates from mesothelial cells and for which diagnosis can be challenging and has a poor prognosis. The diagnosis of MM is mainly based on a combination of histopathologic features, immunohistochemical methods and clinical and imaging information. According to the WHO classification, there are three types of MM: epithelioid, biphasic and sarcomatoid. The epithelioid type of MM exhibits many histological patterns. Because of these features, the differentiation of MM can be a diagnostic challenge, as MM can mimic other cancers. Another important aspect of MM diagnosis is to differentiate from benign proliferations [1–4].

The most common genetic alteration in MM is the deletion of the INK4a/ARF locus, which is located on the short arm of chromosome 9 (9p21). P16 is a cyclin-dependent kinase (CDK) inhibitor that is

Abbreviations: WHO, World Health Organization; MM, malignant mesothelioma; FISH, fluorescence in situ hybridization; TMA, tissue microarray; H&E, hematoxylin and eosin; pT, primary tumor; N, lymph node involvement; M, distant metastasis

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encoded by the INK4a/ARF locus. The INK4a/ARF locus also encodes p14ARF, a protein responsible for MDM2 (murine double minute 2) degradation, which results in p53 dysfunction, rapid cell growth and cell immortality, leading to malignancy [5–9]. Although homozygous deletion of 9p21 is very specific, it is not detected in all MMs. As reported recently by other investigators, the detection of homozygous 9p21 deletion using a FISH technique is useful for distinguishing benign proliferations from MMs.

To interpret FISH signals, a cut-off value for each probe should be determined for a correct diagnosis. To our knowledge, there is no consensus or confirmed protocol for cut-off values to evaluate FISH signals in MMs. Previous studies define homozygous deletion if both 9p21 signals were lost in at least 20% of the nuclei and the cells showed at least one signal for the CEP-9 probe, but previous studies did not declare how the percentage was determined [10–16]. Our study addresses 9p21 deletion status and p16 expression profiles of MM by determining our own cut-off values and the effectiveness of using p16 negativity and 9p21 deletion as markers for differentiating MMs from benign mesothelial proliferations. Because the patients who have 9p21 homozygous deletion are also p16 negative, we concluded that p16 negativity can be used to as a surrogate marker for determining the applicability of the FISH method.

2. Materials and methods

Seventy-four patients who were diagnosed with MM between 2004 and 2014 at the Department of Pathology, Faculty of Medicine, Gazi University, and 40 patients who were diagnosed with MM between 2009 and 2014 at the Atatürk Chest Diseases and Thoracic Surgery Education and Research Hospital were studied. The diagnoses were based on morphology in hematoxylin and eosin (H&E)-stained sections and confirmed using immunohistochemical methods [3,17]. Clinical information was gathered by using the institutes' database records.

The median age of the patients was 58.37 ± 13.05 years. There were 65 men (57.1%) and 49 women (42.9%). All of the specimens were pleural biopsies or surgical resections.

H&E-stained slides were reviewed by two pathologists (GK and LM) and classified as epithelioid, sarcomatoid, or biphasic MM.

It is a well known fact that there is heterogenity in MM. Since Chiosea et al showed no difference in FISH results between whole tissue sections and tissue microarrays (TMAs), we used TMAs in our study one core per each case [14]. Representative areas rich in tumor cells were identified and outlined on each H&E-stained slide and the tissue samples were removed from formalin-fixed paraffin-embedded archival blocks by using a 5-mm punch biopsy tool. Then, the selected samples measuring 5 mm in diameter were inserted into a handmade TMA tray that had 4 rows and 5 columns. Lastly, the cores within the tray were inserted into a recipient paraffin tissue array that contained 20 samples in each block. A map was prepared to label which area represents which case. We monitored the biphasic MMs to include both sarcomatoid and epithelioid areas. One core for each case was used. TMAs were sliced 4 μm thick sections.

2.1. Immunohistochemical staining

The immunohistochemical study was performed using an anti-p16 mouse monoclonal antibody (CINtec, clone E6H4) and Ventana-XT automated slide stainer according to a standard avidin-biotin-peroxidase complex method. Nuclear staining with or without cytoplasmic staining or only cytoplasmic staining was negative in < 10% of tumor cells (Fig. 1; [18]. Staining between 10–33% was considered (+1) positive, 34–65% was (+2) positive and > 66% was (+3) positive (Fig. 2). A cervical carcinoma biopsy sample known to be positive for the p16 protein was used as a positive control.

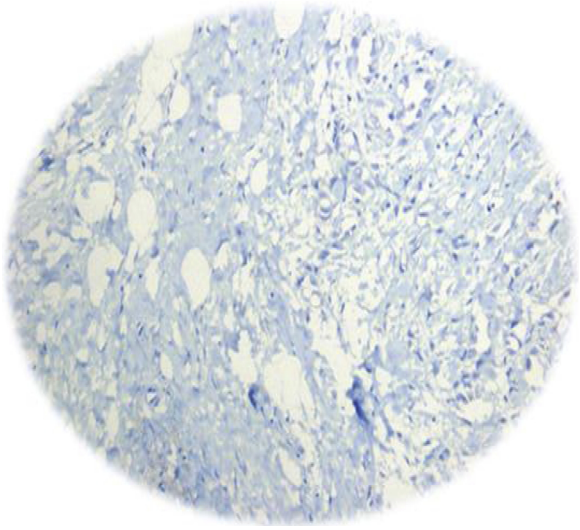

Fig. 1. Tumor cells showing loss of p16 protein expression (IHC 200X).

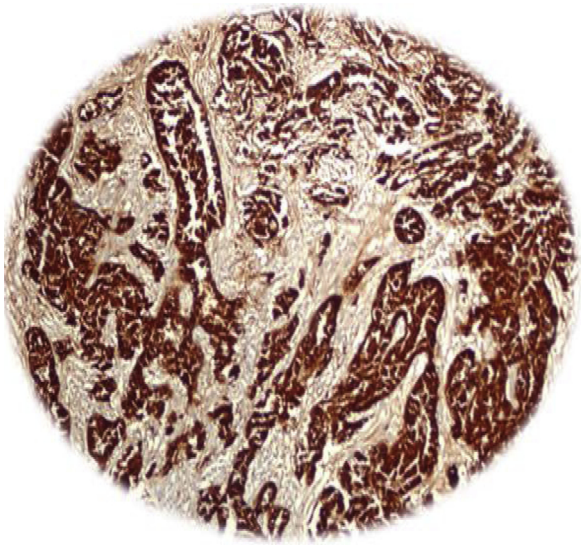

Fig. 2. Tumor cells that are diffusely p16 positive (IHC 200X).

2.2. Fluorescence in situ hybridization (FISH) analysis

FISH analysis was performed by using the Vysis LSI CDKN2A Spectrum Orange/CEP 9 Spectrum Green Probes and the Paraffin Pretreatment Kit IV. The probes contain genetic loci including D9S1749, DS1747, p16 (INK4B), p14 (ARF), D9S1748, p15 (INK4B), and D9S1752. The slides were evaluated by using a BX51 Olympus fluorescence microscope and counting at least 100 nonoverlapping tumor cells.

In a normal cell, two p16/CDKN2A (9p21) signals (orange) and two CEP-9 signals (green) with a dual color FISH probe can be observed (Fig. 3). Homozygous deletion is defined as the absence of both 9p21 signals in the nuclei of a tumor cell and at least one signal for the CEP-9 probe (green; Fig. 4). To interpret FISH signals, a cut-off value for each probe should be determined for a correct diagnosis [19]. To our knowledge, there is no consensus or confirmed protocol for cut-off values to evaluate FISH signals in MMs. Previous studies define homozygous deletion if both 9p21 signals were lost in at least 20% of the nuclei and the cells showed at least one signal for the CEP-9 probe, but previous studies did not declare how the percentage was determined [10–16]. Di Wu et al and Chung et al proposed a cut-off value according to the frequency of 9p21 homozygous deletion obtained from

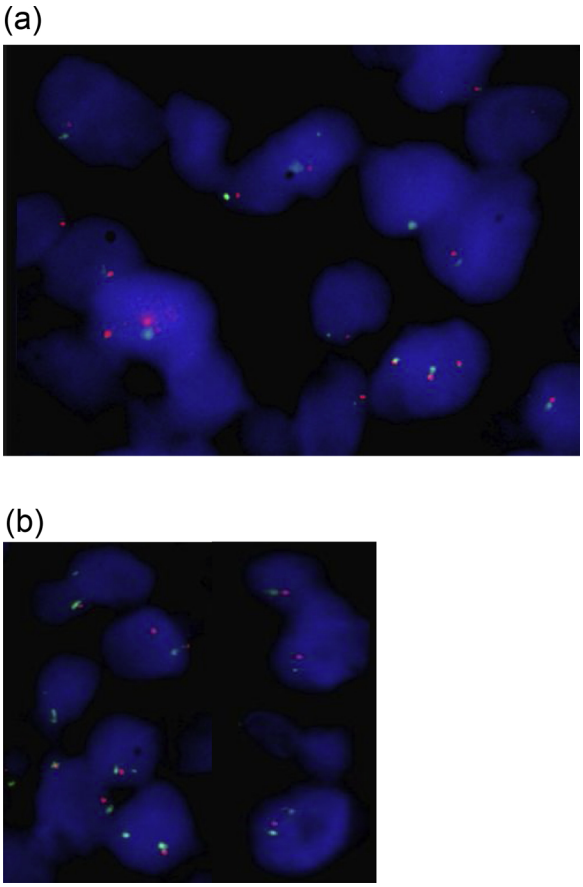

Fig. 3. a Fluorescence in situ hybridization of non-deleted tumor cells (2 red 2 green signals or 1 red 1 green signal).b Fluorescence in situ hybridization of non-deleted tumor cells (2 red 2 green signals or 1 red 1 green signal).

benign controls. The suggestion by Chung et al was 10%, and 14.4% was suggested by Di Wu et al [20,21]. For this reason, we looked for 9p21 deletions in benign proliferations from our archive and found between 0–7%. In our study, we established a cut-off value for the detection of 9p21 deletion by using 13 benign reactive cases (6 reactive mesothelial hyperplasias and 7 chronic fibrinous pleuritis cases). In a standard data distribution, 99.994% of the values were within 4 standard deviations (SDs), so we calculated the cut-off value according to the formula: mean percentage (2.31) + four standard deviations (4 × 2.75). As a result, homozygous deletion was defined by loss of both p16 gene signals in at least 13.3% of the nuclei that showed at least 1 signal for the CEP 9 probe. The cases that showed deletion less than %13.3 accepted as non-deleted. Heterozygous deletions are not the target of this study but hetereozygous deletions were seen between 0–40% in benign proliferations and between 0–55% in MM cases.

3. Statistical analysis

Statistical analyses were performed using the IBM Statistics 20.0 (SPSS) computer program. In statistical analysis, categorical variables were given as numbers and percentages and continuous variables were presented with mean ± standart deviation (SD) and median (min-max value) for descriptive analyses. Chi-square tests were used for comparison of categorical variables between groups. The variables were investigated using visual (histograms, probability plots) and analytical methods (Kolmogorov-Smirnov / Shapiro-Wilk's test) to determine wherher or not they are normally distributed. Independent samples *t*-test was used for comparison of data sets which were normally distributed for the variables between two groups. One way ANOVA test was used to compare the mean between three and more groups. $p < 005$ was considered statistically significant.

4. Results

One hundred fourteen cases of MM were evaluated [74 epithelioid (64.9%), 33 biphasic (28.9%), and 7 sarcomatoid (6.2%)].

Loss of p16 protein expression was detected in 65.8% (75/114) of all cases. Regarding the histological subtypes, p16 was negative in 51.4% (38/74) of epithelioid MM, in 93.9% (31/33) of biphasic MM, and in 85.7% (6/7) of sarcomatoid MM (Table 1). The correlation between p16 protein expression and the histologic subtypes of MM was statistically significant ($p = 0.001$; Table 2). The correlations between p16 protein expression and age, sex, primary tumor (pT) type, lymph node involvement (N) and distant metastasis (M) were statistically insignificant ($p = 0.490$, $p = 0.237$, $p = 0.646$, $p = 0.537$, and $p = 0.457$, respectively). p16 expression of pT4 (83.3%), N3 (100%) and M1 (87.5%) tumors were highly negative; therefore, this result can be useful for predicting prognosis.

FISH results demonstrated 9p21 deletion in 82 of 114 cases (71.9%). Homozygous deletion was noted in 44 of 74 (59.5%) epithelioid MMs, 31 of 33 (93.9%) biphasic MMs and 7 of 7 (100%) sarcomatoid MMs. The correlation between 9p21 homozygous deletion and histological subtype of MM was statistically significant ($p < 0.001$; Table 3). The deletion was more common in cases of sarcomatoid MM (100%) than those of epithelioid MM (59.5%) or biphasic MM (93.9%), consistent with other studies. The age of the patients was higher in 9p21 deleted cases, and this result was statistically significant ($p = 0.043$). The correlation between 9p21 deletion and sex, pT, N and M was

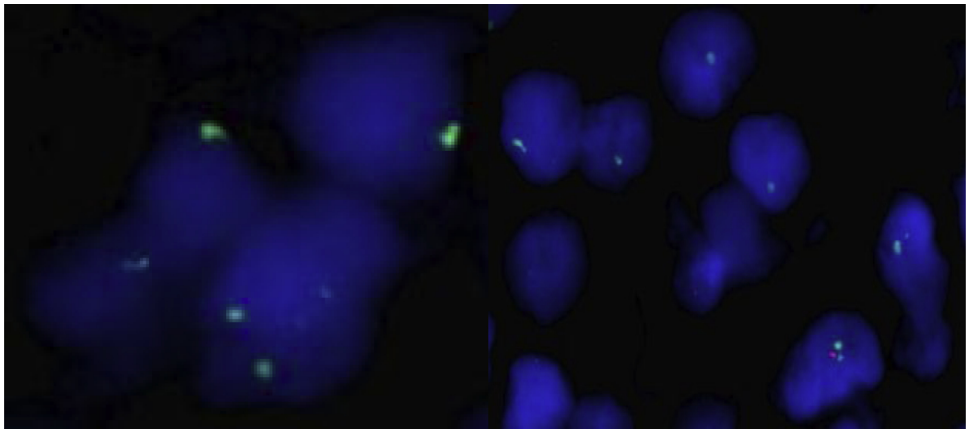

Fig. 4. Fluorescence in situ hybridization showing homozygous 9p21 state (loss of both red signals).

Table 1
Frequency of p16 expression based on the histologic subtypes of MM.

p16 expression		Frequency	%
Epithelioid	Negative	38	51,4
	Positive	36	48,6
Biphasic	Negative	31	93,9
	Positive	2	6,1
Sarcomatoid	Negative	6	85,7
	Positive	1	14,3

Table 2
The correlation between p16 protein expression and the histologic subtypes of MM.

		Histologic Subtype			p
		Epithelioid	Biphasic	Sarcomatoid	
p16 expression	Negative	38	31	6	0,001
		50,7%	41,3%	8,0%	
	Positive	36	2	1	
		92,3%	5,1%	2,6%	
Total		74	33	7	114
		64,9%	289%	6,1%	100,0%

Table 3
Frequency of 9p21 deletion based on the histologic subtypes of MM.

		Histologic Subtype		p
		Non-deleted	Deleted	
Histologic subtype	Epithelioid	30	44	< 0,001
		40.5%	59.5%	
	Biphasic	2	31	
		6.1%	93.9%	
	Sarcomatoid	0	7	
		.0%	100.0%	1000%

statistically insignificant ($p = 0.908$, $p = 0.411$, $p = 0.378$, and $p = 1.000$, respectively).

The correlation between 9p21 deletion and pT, N or M was statistically insignificant, but 100% of pT4 tumors, 100% of N3 tumors and 70% of M1 tumors exhibited 9p21 deletions; therefore, this result can be useful for predicting prognosis. These results are consistent with loss of p16 protein expression.

The correlation between p16 protein expression and 9p21 homozygous deletion in the tumor cells was statistically significant ($p < 0.001$; Table 4). It was detected that in 86.7% (65/75) of cases that are p16 negative exhibit the 9p21 homozygous deletion hence, if p16 is positive, the probability of having the homozygous deletion is decreased. The percentage of homozygous or heterozygous deletions were not related with p16 negativity therefore this cann't be identified with the heterogenity of tumor. The sensitivity of 9p21 deletion was 71.9%, and the specificity was 100%. The sensitivity of loss of p16 expression was 74.5%, and the specificity was 50%.

Table 4
Relationship between p16 protein expression and 9p21 homozygous deletion.

		9p21 FISH		p
		Non-deleted	Deleted	
p16 expression	Negative	10	65	< 0,001
		13,3%	86,7%	
	Positive	22	17	
		56,4%	43,6%	
Total		32	82	114
		28,1%	71,9	100,0%

5. Discussion

MM is an aggressive and rare cancer that has many morphological features and diagnostic challenges and has an increasing incidence, especially in developed countries [22–24]. It is essential for patient treatment and prognosis to differentiate between benign proliferations and MM. There is not an ancillary technique that is 100% specific or sensitive for MM; therefore, differentiation of benign proliferations from MMs can be difficult to achieve.

In the last decade, there have been several studies that analyzed 9p21 deletion in MMs. According to these studies, 9p21 deletion occurs in 55.6–88% of epithelioid MM cases, in 62.5–87.5% of biphasic MM cases, and in 100% of sarcomatoid MM cases [10,11,14,21,25]. Likewise, our study corroborated these literature values. Deletion of 9p21 was identified in 59.5% of epithelioid MM cases, in 93.9% of biphasic MM cases, and in 100% of sarcomatoid MM cases. We think that the discrepancy between studies can result from using different cut-off values.

To our knowledge, there is no consensus or confirmed protocol for cut-off values to evaluate FISH signals in MMs. Previous studies define homozygous deletion if both 9p21 signals were lost in at least 20% of the nuclei and the cells showed at least one signal for the CEP-9 probe, but previous studies did not declare how the percentage was determined [10–16,20]. Di Wu et al and Chung et al proposed a cut-off value according to the frequency of 9p21 homozygous deletion obtained from benign controls. Di Wu et al stated that homozygous deletion pattern was noted in 0 to 7.4% of benign proliferations. Similarly we found 0 to 7% homozygous deletion.

The suggestion for cut-off value by Chung et al was 10%, and 14.4% was suggested by Di Wu et al [20,21]. Because of the fact that we detected 9p21 deletions in benign proliferations a cut-off value for each probe should be determined according to the frequency of 9p21 homozygous deletion obtained from benign controls to interpret FISH signals.

In our study, the correlation between histological subtypes and 9p21 deletion was statistically significant; on the other hand, Takeda et al and Illei et al did not find a correlation [10,11,14]. These studies determined that 9p21 deletion was more common in cases that have sarcomatoid elements. In their studies, there were few biphasic and sarcomatoid MM cases, and the FISH method was performed using different cut-off values; this could be the reason why they could not detect a correlation. According to our results, homozygous deletion was noted in all sarcomatoid MM cases and in none of the fibrous pleuritis cases (more than 13.3%). All these results indicate that 9p21 FISH analysis of MM specimens seems to be useful for diagnostic purposes, especially in sarcomatoid MM [20,21]. Diagnosis of sarcomatoid MM is a challenge for pathologists, the reason is that many ancillary markers used for epithelioid MM are not useful in sarcomatoid type. Therefore FISH technique can be a usefull diagnostic tool for especially sarcomatoid MM.

There are limited data that demonstrate loss of p16 protein expression by immunohistochemistry. Our study demonstrated loss of p16 protein expression in 75 of 114 MM cases (65.8%). Loss of p16 expression was found in 51.4% of epithelioid MM, 93.9% of biphasic MM, and 85.7% of sarcomatoid MM. Loss of p16 protein expression was observed in 40–85% of cases in other studies [11,13,14]. The correlation between loss of p16 protein expression and 9p21 deletion was statistically significant ($p < 0.001$) in our study, as in the studies by Takeda et al, Krasinskas et al and Dacic et al [11,13,26]. Among the cases that are p16 negative we detected that 86.7% also have the 9p21 deletion.

There was no correlation between p16 expression in histologic subtypes of MM and pT, N or M status. Despite statistical insignificance, pT4 (83.3%), N3 (100%) and M1 (87.5%) tumors were highly negative for p16. All of these results suggest that loss of p16 expression is related to a worse prognosis and, besides diagnostic purposes, can be useful for

predicting a prognosis [13]. Likewise, Kobayashi et al found that patients who are p16-positive live longer than p16-negative patients among Japanese population [6]. Additionally, Dacic et al showed p16-positive immunoexpression can significantly predict long-term survival [13]. The combined detection of 9p21 deletion and loss of p16 expression is helpful for diagnostic purposes, but because the FISH method is an expensive technique and loss of p16 expression is not specific for mesotheliomas, p16 negativity can guide practitioners as a surrogate marker to eliminate cases that require further investigation by FISH.

While much of the literature on 9p21 deletion in MMs has limited cases, to our knowledge our study includes the largest number of patients. In addition, only two studies on this subject in the literature established their own cut-off values. Likewise, our study was developed by determining our own cut-off values with widest patient number. The variability in 9p21 homozygous deletion results from different institutes suggests that each institute should establish its own cut-off value by using reactive mesothelial proliferations, or we need further global research studies to assess cut-off values. Diagnosis of MM is challenging not only in biopsies but also in effusions and small tissue samples; thus, loss of p16 protein expression combined with 9p21 deletion detection can be used to distinguish between MMs and reactive proliferations [27].

6. Conclusion

According to literature it's confirmed that 9p21 homozygous deletion is a valuable technique for diagnostic and prognostic purposes in MM. However results differ between studies and there is no confirmed protocol for cut-off values to evaluate FISH signals in MMs. İn our study homozygous deletion was noted in 0–7% of benign proliferations which indicates that a cut-off value needs to be calculated by using reactive proliferations or we need further global research studies to assess cut-off values. Diagnosis of MM is a challenge for pathologists particularly for sarcomatoid type. Therefore FISH technique can be a usefull diagnostic tool for especially sarcomatoid MM. The prevalence of 9p21 deletion is very high in MMs, this knowledge can be used for therapeutic methods in the future, such as for lung carcinomas, in addition to the diagnostic usefulness.

Since FISH probes for 9p21 are analyte-specific reagents (ASRs) whose performance characteristics are determined by each laboratory, validation studies approved by FDA are needed for therapeutic methods in the future.

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