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LABORATORY INVESTIGATION

1P19Q loss but not *IDH1* mutations influences WHO grade II gliomas spontaneous growth

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Abstract Mutations at the codon 132 in the isocitrate dehydrogenase 1 (*IDH1*) gene occur early, with a high frequency, in World Health Organization (WHO) grade II gliomas. We investigated the impact of *IDH1* mutations on spontaneous glioma growth rate, known to be an early prognostic factor.The mean tumor diameter was assessed on the first MRI performed at diagnosis and on a second MRI, performed immediately before surgery, in a series of 64 WHO grade II gliomas. The patients did not undergo treatment before surgery. Because of a frequent association, we jointly analyzed the 1p19q co-deletion and *IDH1* mutations effects on tumor velocity of diameter expansion (mm/year) during preoperative spontaneous growth period. 1p19q co-deletion had a significant slowing effect ($p = 0.0133$) on tumor growth estimated at -1.7760 ± 0.711 mm/year (95% CI -3.154, -0.366), whereas *IDH1* mutations estimated effect of $+0.036 \pm 0.833$ mm/year (95% CI -1.668; $+1.596$) was not significant ($p = 0.9654$). Our results provide first evidence that *IDH1* mutations are not significantly involved in tumor growth rate. By contrast, we confirm that 1p19q co-deletion decreases growth velocity.

Keywords IDH1 · 1p19q · WHO grade II glioma · Spontaneous growth · Prognosis

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Introduction

Recent sequencing of the genome of glioblastomas identified novel mutations in the isocitrate dehydrogenase 1 gene *(IDH1)* in the highly conserved residue R132 [1]. The highest rates of *IDH1* mutations, comprised between 73 and 100% depending on the series, have been reported in World Health Organization (WHO) grade II gliomas [2–4]. Watanabe et al. [3] showed that *IDH1* mutations occur very early in low-grade gliomas.

WHO grade II gliomas (GIIG) are diffuse infiltrating tumors which inexorably progress to high grade. The timing of their evolution is quite unpredictable, making early prognostic markers essential to improve the therapeutic management. Until now, the prognosis value of *IDH1* mutations was only investigated with survival as an end point [5–8]. In GIIG, *IDH1* mutations were proved to have significant impact on overall survival only (OS). OS does not take into account the heterogeneity of therapeutic schemes experienced by the patients. This is all the more important as no consensus therapeutic management has

been validated to date in GIIG. Finally, OS in GIIG can be as long as 15 and even 20 years. Therefore, OS is an inappropriate way to evaluate the impact of a molecular event on the first steps of a spontaneous tumor course. In contrast, the tumor growth kinetics directly evaluates the tumor behavior. The tumor growth is a continuous process [9], and a prognosis value has been assigned to growth rate [10]. When assessed before any treatment, including surgery, any influence of treatment is ruled out.

1p19q co-deletion is an independent favorable prognostic factor on OS as reported by many studies [11–17]. Moreover, a preliminary study on a short series showed an effect of 1p19q loss on GIIG spontaneous growth [18]. In addition, Labussiere et al. [19] reported that 1p19q complete deletion and *IDH1*/*IDH2* mutations are closely associated. Thus, ignoring the effect of 1p19q on tumor growth rate while estimating the effect of IDH1, or vice versa, could lead to serious bias. Instead, we assessed the combined effects of *IDH1* mutations and 1p19q loss on spontaneous tumor growth kinetics with a linear model.

Materials and methods

Patients

Patients were selected according to the following criteria: histological diagnosis of WHO grade II glioma, no treatment before surgery, and measurable tumor disease on at least two serial magnetic resonance imaging (MRI) examinations. The first MRI was performed at diagnosis and the second immediately before surgery in order to assess the tumor kinetics during the whole spontaneous growth period. The two MRIs were spaced by at least 3 months. Frozen tumor and blood DNA were available. Patients gave written informed consent. Sixty-four patients were eligible for this study.

Mean tumor diameter (MTD) estimation and evaluation of the MTD slope

The tumor volume was calculated on the basis of the three largest diameters (D1, D2, D3) on FLAIR or T2-weighted signal abnormalities according to the three orthogonal planes (axial, sagittal, and coronal). An estimation of the tumor volume was calculated by the ellipsoid approximation [$V = (D1 \times D2 \times D3)/2$] as described by Mandonnet et al. [9]. Volumes were converted into Mean Tumor Diameter [$MTD = (2 \times V)^{1/3}$]. The Velocity of Diameter Expansion (VDE) was evaluated by the mean annual growth rate (mm/year) between the two MRI measurements.

Molecular biology

Areas with tumor cell content superior to 60% were selected by pathologist examination. Tumour DNA was isolated according to a salting-out procedure [20]. Blood DNA was extracted from the patient's EDTA peripheral blood using the MagNA Pure Compact robot (Roche Diagnostics, Mannheim, Germany).

1p19q Loss of heterozygosity

Blood and tumour DNA were genotyped for a panel of highly polymorphic microsatellite markers: on 1p (D1S2660, D41S450, D1S507, D1S234, D1S2890, D1S230, D1S207, D1S206) and 19q (D19S414, D19S420, D19S903, D19S571) provided by the ABI Prism Linkage Mapping Set 2.5 (Applied Biosystems, Foster City, USA).

IDH1 mutation screening

A fragment of 254 base pairs (bp) length spanning the catalytic domain of *IDH1* including the codon 132 was amplified using the sense primer *IDH1*f 5′-ACCAAATGGCA CCATACGA-3′ and antisense primer *IDH1*r 5′-TTCATAC CTTGCTTAATGGGTGT-3′ in PCR conditions described by Balss et al. [21]. For confirmation, a 129-bp fragment was amplified by using the sense primer *IDH1*f' 5′-CGGTCTTC AGAGAAGCCATT-3′ and the antisense primer *IDH1*r' 5′-GCAAAATCACATTATTGCCAAC-3′ at the same conditions as for the first primers set. After purification (multiscreen PCR plates; Millipore, Corrigtwohill, Co.Cork, Ireland), the 254-bp fragments were submitted to the sequencing reaction using the Big Dye Terminator v 1.1.sequencing kit (Applied Biosystems, Foster City, USA) with the sense primer *IDH1*f. A second round of sequencing using the antisense primer *IDH1*r′ was performed on the 129-bp fragment in each case of unclear sequences for codon 132.

Immunohistochemistry for p53 and Ki67 expression detection on paraffin-embedded tissue

Since overexpression of p53 protein reflects to some extent the *TP53* gene mutations status, we used protein immunostaining as a handing practice to evaluate the mutation rate of the *TP53* gene [22–25]. Antigen retrieval for p53 and Ki67 staining was done using EDTA, pH 7–8. Monoclonal antibodies for p53 (Ménarini Diagnostics, Florence, Italy) and Ki67 (Dako, Glostrup, Denmark) were used at dilutions 1:40 and 1:100, respectively. Labeled streptavidin biotin kit was used as detection system (Benchmark Ultra; Ventana Medical Systems, Tucson, AZ, USA). For p53, cases where more than 10% of nuclei showed positivity were considered

as positive. Immunopositive cases were graded from 1+ to 3+ as follows: 1+ positivity: 11–33% cells stained; 2+ positivity: 33–66% cells stained; 3+ positivity: 66–100% cells stained. For Ki67 staining, areas of highest cellularity were used for quantization. The percentage of labeled cells was determined from a count of 100 cells. The retained Ki67% labeling was assessed in the area with the highest level of proliferation.

Statistical analysis

Pearson's Chi-square and Fisher's exact tests were used to relate *IDH1* mutations status with other molecular characteristics, histological type, localization and volume at diagnosis of the tumors and age of the patients, considering two groups in each case. Chi-square was performed when all the expected cell counts under null hypothesis were at least 5. Otherwise, Fisher's exact test was performed instead. We then studied the simultaneous influence of *IDH1* mutation and 1p19q deletion on the glioma growth rate. Separating between the effects of two or more factors influencing a common factor has been elucidated by Fisher [26] with the analysis of variance of factorial experiments, and its scope could be extended by Yates [27, 28] to unbalanced data. This is the case here as 1p19q deletion and IDH1 mutation are not independent.

Tumor growth was supposed to follow the standard linear model for a two-way analysis of variance without interaction; that is to say, the effects of IDH1 mutation and 1p19q mutation are supposed to add up:

$$Y_i = m + a1_{IDH1}(i) + b1_{1p19q}(i) + E_i$$

where Y_i is the glioma growth rate of patient i, a is the effect of *IDH1* mutation, b is the effect of 1p19q deletion, 1_{IDH1} (i) is the indicator of an *IDH1* mutation on patient i, 1_{1p19q} (i) is the indicator of a 1p19q deletion on patient i, and E_i is a random variation.

Because of the presence of several outliers in the growth rates, we replaced the analysis of variance for this model with a robust method, the M-estimation [29] that is not sensitive to a reasonable number of outliers. This M-estimation was carried out using the Robustreg procedure of the version 9.2 of SAS software (SAS Institute, Cary, NC, USA).

Results

Patient demographics and clinical characteristics

All the 64 patients included in this study underwent surgery for a WHO grade II glioma. No chemotherapy or radiotherapy was performed before surgery in this series. The sex ratio was 1.78 (41 men and 23 women). The median age at diagnosis was 36.5 years and the median follow-up duration of spontaneous growth was 10 months. The median tumor volume evaluated on the MRIs performed at diagnosis was 39.5 cm^3, while it was 53.5 cm^3 on immediate preoperative MRI. No contrast enhancement was seen in any of the tumors. The median tumor growth kinetics (VDE) was 3.5 mm/year in accordance with other reports [9, 10, 30]. A summary of patient clinical data is given in Table 1.

Table 1 Characteristics of patients ($n = 64$)

Characteristics	Value	Range
Sex		
Male	41	
Female	23	
Male to female ratio	1.78	
Median age at diagnosis (years)	36.5	18–64
Median tumor volume (cm^3)		
At diagnosis	39.5	1–173
Preoperative	53.5	7–220
Median follow-up duration (months)	10	3–152
Median tumor growth kinetics (mm/year)	3.52	0–24.33

Histological and molecular characteristics of the tumors

The distribution of histological subtypes was balanced in our series: 33% of oligodendrogliomas (21/64), 33% of oligoastrocytomas (21/64), and 34% of astrocytomas (22/64). *IDH1* mutations were present in 52 (81%) of the 64 patients. The most frequently found mutation of *IDH1* gene was G395A (Arg132His) retrieved in 85% (44/52) of the cases. In other cases, mutations occurred at C394 nucleotide with a C > G (4/52, 8%), a C > T (2/52, 4 %) or a C > A (2/52, 4%) changes. *IDH1* mutations were mainly associated with oligodendrogliomas (95% of mutation rate). The *IDH1* mutations were less frequent in oligoastrocytomas and astrocytomas (80 and 68%, respectively); however, this difference did not reach significance (Fisher's exact test, $p = 0{,}067$). Thirty percent (19/64) of the tumors displayed a complete 1p19q co-deletion. As expected, 1p19q complete co-deletion was more frequent in oligodendroglioma than in oligoastrocytoma and astrocytoma (χ^2 test, $p < 0{,}001$). In the same way, p53 overexpression was more frequent in both oligoastrocytomas and astrocytomas than in oligodendrogliomas (χ^2 test, $p < 0{,}001$), being found in 54% (32/60) of all tumors and with a higher frequency of 70% in both oligoastrocytomas and astrocytomas.

The data are summarized in Table 2.

Table 2 Correlation of molecular and histological patterns of the tumors

Genetic alteration	Oligodendroglioma ($n = 21$)	Oligoastrocytoma ($n = 21$)	Astrocytoma ($n = 22$)	Total ($n = 64$)
1p19q				
Complete co-deletion	17 (71%)	4 (19%)	0	19 (30%)
Others	6 (29%)	17 (81%)	22 (100%)	45 (70%)
IDH1 mutation				
Mutated	20 (95%)	17 (80%)	15 (68%)	52 (81%)
Non-mutated	1 (5%)	4 (20%)	7 (32%)	12 (19%)
P53 expression				
0	17 (81%)	6 (30%)	5 (26%)	28 (46%)
+ to +++	4 (19%)	14 (70%)	14 (74%)	32 (54%)
ND	0	1	3	4

ND not measured

Correlation of *IDH1* mutation status with clinical and molecular characteristics of tumors

Comparison of IDH1 mutations rate in tumors with or without 1p19q complete co-deletion or in tumors with or without p53 overexpression did not show statistically significant differences. The insula was the most frequent brain location of our series nevertheless the *IDH1* mutation rate was not significantly dependent on location (χ^2 test $p = 0.15$). *IDH1* mutations had no influence on tumor volume or age of the patients at diagnosis. All the data and statistical analyses relative to correlation of *IDH1* mutation status and tumors characteristics are compiled in Table 3.

Table 3 Correlation of IDH1 mutations status and histological, topographic, molecular and presentation at diagnosis characteristics of tumors

	IDH1 Mutated/total	% Mutation	p^a
Histological subtype			0.067
Oligodendroglioma	20/21	95	
Others	32/43	74	
Brain location			0.151
Insula	14/20	70	
Others	37/42	88	
1p19q			0.484
Complete co-deletion	17/19	89	
Others signatures	35/45	77	
p53 expression			1
0	23/28	82	
+ to +++	27/32	84	
Ki67% immunopositivity			0.475
≤5%	32/41	78	
>5%	17/19	89	
Tumor Volume at diagnosis			1
<median (39.5 cm^3)	26/32	81	
>median (39.5 cm^3)	26/32	81	
Age at diagnosis			0.521
<median (36.5 years)	27/32	84	
>median (36.5 years)	25/32	78	

[a] p values were calculated by the Chi-square or Fisher's exact test: Chi-square was performed when all the expected cell counts under null hypothesis were at least 5, otherwise Fisher's exact test was performed

Combined effect of *IDH1* mutations and 1p19q complete deletion on VDE

The 1p19q co-deletion effect on growth rate was estimated at -1.7760 ± 0.711 mm/year (95 % CI -3.154, -0.366), thus significantly decreasing the effect ($p = 0.0133$). By contrast, the effect of *IDH1* mutations on growth rate was not significant ($p = 0.9654$): it was estimated at $+0.036 \pm 0.833$ mm/year (95% CI -1.668; $+1.596$). A scatter plot of VDE (mm/year) versus genotype is shown in Fig. 1 together with the means and SEM of VDE in each of the four groups.

Discussion

The prognosis impact of *IDH1* mutations has been investigated in several studies with a reported favorable effect on glioma outcome [5, 7, 8]. Conversely, Kim et al. [6], on the largest series of WHO grade II gliomas studied to date, reported a conflicting result: *IDH1/2* mutations were devoid of significant effects on prognosis in their study. All these studies were conducted with overall survival and progression-free survival as main assessment criteria.

Until now, no study has focused on the spontaneous tumor growth rate (TGR). TGR is a direct way to estimate tumor behavior by exempting evaluation from treatment influences. Previously, a prognosis value has been demonstrated for mean tumor diameter assessment over time [10]. We used the dynamic analysis of WHO grade II gliomas growth to evaluate the impact of genetic changes occurring during gliomagenesis. In the same experimental conditions, considering the

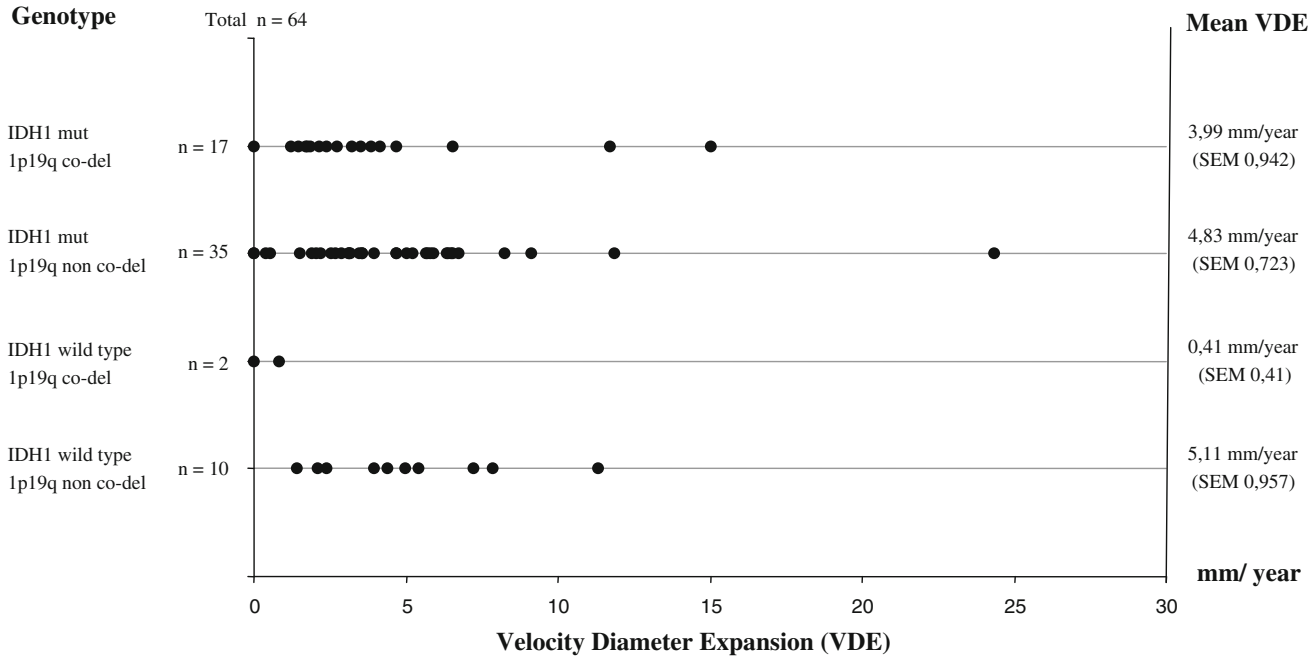

Fig. 1 Tumors have been divided into four groups according to IDH1 mutation (mutated, wild-type) and 1p19q (complete co-deletion, no complete co-deletion) genotypes. No complete 1p19q co-deleted tumors are tumors with partial deletion or no deletion in 1p and 19q chromosomes. The mean annual growth rate for each group is reported with corresponding standard error of the mean (SEM)

respective influence of each factor by means of a robust two-way analysis of variance, 1p19q co-deletion slowed down the tumor growth while *IDH1* mutations did not have a significant effect. The significant effect of 1p19q deletion did not appear in *t* test group mean comparisons (see Fig. 1 group means and SEM), as this method is less powerful than the proper two-way analysis of variance, and the power is further impaired by the outliers which conversely do not influence the M-estimation. Limitations of the study are the size of the series ($n = 64$ patients) and the time interval of follow-up between the two serial MRIs (median 10 months). A larger series could have allowed the detection of a possible slight effect of IDH1 mutations on tumor growth. Concerning spontaneous growth follow-up duration, it is difficult to increase its length because the present therapeutic management of low-grade gliomas has moved from a "wait and see" attitude towards an early treatment implementation.

Recently reported studies confirm our results concerning IDH1 mutations effects: in the work of Kim et al. [6], using overall survival as prognosis indicator, IDH mutations did not probe to improve survival. A recent work from Houllier et al. [7] reported a questionable effect of *IDH1/2* mutations on tumor spontaneous re-growth after surgery in patients who did not have adjuvant chemotherapy or radiotherapy. Even though surgery is known to have an actual impact on natural history of gliomas [31], this finding relating to re-growth in the absence of treatment trends towards the same results as ours on spontaneous preoperative growth.

For 1p19q co-deletion, our data confirm, but in a three-fold larger homogeneous series, previous findings [18]. In that study, the reported mean tumor growth rate was 3.4 mm/year in 1p19q co-deleted tumors versus 5.9 mm/year in non-deleted 1p19q tumors ($p = 0.0016$). So, if 1p19q co-deletion and slower natural progression of disease are linked together, it could be anticipated that 1p19q status is a prognosis factor per se. Taking into account the 1p19q co-deletion effect on tumor growth might provide some help in deciphering the intricate effects of 1p19q co-deletion on favorable outcome: is it an intrinsic prognosis factor or a chemosensitivity marker?

A high degree of association between *IDH1/2* mutations and 1p19q complete co-deletion has been reported by several studies [7, 8, 19]. It was reported as a systematic association in one of these studies mixing gliomas from all WHO grades [19]. However, in our series, some of the 1p19q co-deleted tumors were not IDH1 mutated. It is noteworthy that we did not perform *IDH2* gene mutations research. *IDH2* mutations are almost never associated with *IDH1* mutations and retrieved rarely in about 2–3% of gliomas [4]. In a series of 360 low-grade gliomas, Kim et al. [6] retrieved 10% of 1p19q co-deleted tumors without IDH1 or IDH2 mutations. So, IDH2 mutations screening would possibly have increased the number of 1p19q co-deleted tumors harboring mutated *IDH* genes, but very little. Because of the strong association between these two molecular hallmarks, the prognosis value of each of them could interfere with the other. We thus performed a

two-way analysis of variance with *IDH1* mutations and 1p19q co-deletion as co-factors to eliminate 1p19q loss influence. With regard to spontaneous tumor growth rate, 1p19q loss had an effect whereas *IDH1* mutations did not. On overall survival, Sanson et al. [5] showed a favorable effect of *IDH1* mutations and 1p19q co-deletion in a study with numerous factors. In order to eliminate the influence of 1p19q co-deletion in their study of IDH mutations impact on overall survival, Metellus et al. [8] confirmed the first results obtained on the whole series by performing a second statistical analysis only on 1p19q non-co-deleted tumors. But then the size of the series restricted to 1p19q non-co-deleted tumors was greatly reduced, decreasing the statistical power.

IDH1 enzyme is involved in a fundamental metabolic pathway converting isocitrate, from the tricarboxylic acid cycle, in α-ketoglutarate. The codon 132 *IDH1* gene mutations impair the enzymatic activity with a decrease of α-ketoglutarate and an induced illicit increase of 2-hydroxyglutarate levels [32]. These metabolic disruptions have many presumed consequences. Very recently, a functional study of metabolic impairments resulting from IDH1 mutations showed that they affected the in vivo activity of α-ketoglutarate-dependent dioxygenases such as histone demethylases and TET 5-methylcytosine hydroxylases [33]. Xu et al. concluded that consequential alteration of histone and DNA methylation may contribute to tumorigenesis through altering epigenetic control and the fates of stem or progenitor cells. This is in accordance with a previous report of Noushmer et al. that described a CpG island methylator phenotype displaying a genome-wide hypermethylation pattern and defining a distinct subgroup of glioma tightly associated with IDH1 mutations. Patients harboring gliomas of this subgroup experienced a significantly improved outcome [34]. The epigenetic landscape of cancer cells is profoundly distorted. These epigenetic alterations have a crucial early role in cancer spread because they might determine subsequent genetic changes. Therefore, the hypothesis of IDH1 mutations creating metabolic disturbances with widespread repercussions on epigenetic and secondarily on genetic programs of the tumor cell would fit with favorable prognostic impact of these mutations in spite of a lack of effect on spontaneous tumor growth.

In a preliminary study, Push et al. [35] described a secondary glioblastoma resulting from the malignant transformation from an *IDH1* R132H-mutated WHO grade II astrocytoma in which the mutated *IDH1* R132H allele failed to be detected by immunochemistry. This suggests that *IDH1* R132H mutation is not essential to maintain malignant phenotype at least in late stages. This observation reinforces the hypothesis that *IDH1* mutations do not play an essential role in tumor growth. In spite of the loss of IDH1 R132H mutation, the tumor outburst occurred, leading to the ultimate transformation of low-grade glioma into WHO grade IV glioma.

A second hypothesis to explain the reported favorable impact of *IDH* mutations on overall survival could be that the metabolic changes they induce contribute to a more chemosensitive or radiosensitive cellular environment. In a first study, Dubbink et al. [36] showed that *IDH1* mutations had no effect on response to temozolomide. More recently, Houillier et al. [7] reported the opposite result with an association between *IDH* mutations and response to temozolomide.

In summary, this study sheds a new light on *IDH1* mutations and 1p19q co-deletion oncological consequences. Whereas we confirm that 1p19q loss displays a slowing effect on tumor spread in good accordance with a direct favorable impact of this chromosomal deletion on tumor natural history, we provide first evidence that *IDH1* gene mutations do not have a sizable correlation with tumor growth. As a consequence, IDH1 mutations because of their precocity might be considered instead as a causative link between early cellular metabolism disturbances and the emergence of driving molecular events in gliomagenesis.

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