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Differential Immunoexpression of *BRAF/V600E*, Senescence Markers, PTEN, and T-type Calcium Channels in Acquired Naevi According to their Histopathological and Dermoscopic Classification

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BRAF/V600E mutation and other cell growth/growth-control mechanisms are involved in naevogenesis and melanomagenesis. Immunoexpression of BRAF/V600E and other molecules (p16, phosphatase and tensin homologue (PTEN), Ki67, hTERT and Cav3.1 and 3.2 calcium channels) were investigated in 80 histopathologically and dermoscopically classified acquired naevi. Regarding BRAF/V600E, dysplastic naevi showed lower immunostaining than common naevi, which was significant in comparison with intradermal naevi, which showed the highest BRAF/V600E histoscore. Junctional naevi showed the lowest BRAF/V600E levels. Globular/cobblestone and reticular dermoscopic patterns were consistently associated with high and low BRAF/V600E immunoexpression, respectively, but Zalaudek's peripheral globule pattern (CR/PG) showed the highest BRAF/V600E immunoexpression. Among global patterns, the previously not investigated multicomponent pattern showed the lowest BRAF/V600E immunoexpression. Regarding the remaining biomarkers, new immunohistochemical features were found, in particular p16 and PTEN low expression in multicomponent pattern; and Ki67, hTERT and Cav.3.1 high expression in CR/PG. In conclusion, histopathology and dermoscopy provide complementary information regarding the biology of melanocytic naevi.

Key words: acquired melanocytic naevus; BRAF-V600E; senescence markers; PTEN; T-type calcium channels.

SIGNIFICANCE

Naevi share mutations, such as *BRAF/V600E*, with melanoma, which could explain the initial growth of naevi. However, other mechanisms, such as so-called senescence, preclude successive proliferation of naevus cells. Traditionally, naevi have been classified histopathologically. Recently, the diagnostic method dermoscopy, has introduced a new classification of naevi. This study analysed several molecules (BRAF, phosphatase and tensin homologue, senescence and cell growth/activity markers) in a series of acquired naevi. Naevi were classified following histopathology and 2 different dermoscopic classifications. The distribution of the studied molecules among the different naevus groups showed that some molecular characteristics correlated better with dermoscopic characterization, providing complementary information about naevus biology.

Cutaneous melanoma arises on normal skin or from congenital or acquired melanocytic naevi (1). *BRAF/V600E* mutation stimulates naevogenesis in a majority of naevi, and BRAF/V600E-induced senescence explains cell arrest. Senescence abrogation and additional genetic alterations are involved in malignant transformation (2).

Dysplastic naevus (DN) is a controversial entity. A single DN progresses to melanoma in either a similar or a slightly higher frequency than that of common acquired melanocytic naevi (CAN) (3). There is some controversy (4) regarding whether these lesions share alterations (mutations in *non-V600E/BRAF*, *NRAS* and *TERT* promoter, hemizygous deletion of *CDKN2A* (5)) with CAN and melanoma, as well as upregulation of genes involved in cell proliferation, adhesion, migration, and epidermal/follicular keratinocyte-related genes (3). Moreover, T-type calcium channel (TT-C) expression increases from CAN to DN and melanoma and could be a biomarker of tumour progression (6).

Dermoscopy improves diagnosis, helps to elucidate naevus biology (7) and correlates with molecular features: (*i*) most globular naevi have *BRAF* mutations and few copy number aberrations (4, 8); (*ii*) reticular naevi have *BRAF* and *NRAS* mutations, and balanced loss of tumour suppressors and oncogenes (4, 8); and (*iii*) all naevi with a peripheral globule pattern (CR/PG) have *BRAF* mutations (8). BRAF/V600E immunoexpression (BRAF-IHC) was associated with dermoscopy (presence

of globules vs network) and histopathology (intradermal growth or large naevus nests vs the absence of them) (9). There is scarce data regarding *BRAF/V600E* mutation in naevi with other dermoscopic patterns, such as multicomponent pattern (MP).

Histopathological and dermoscopic classifications correlate, but do not provide the same information (7). Some dermoscopic global patterns of melanocytic lesions (10), such as reticular or globular, are coincidental to dermoscopic Zalaudek's patterns (11), but others attempt to capture other features. For instance, atypia (in the case of MP of pattern analysis) (10) or growth (in the case of CR/PG pattern of Zalaudek classification) (11).

In conclusion, molecular alterations related to oncogenes, senescence, proliferation and others were described in DN (3), and in dermoscopically reticular vs globular naevi (4, 8). However, the distribution of these or other alterations among the main CAN and DN histopathological and dermoscopic patterns has not been specifically addressed.

The aim of this study was to simultaneously investigate the immunoexpression of BRAF/V600E, phosphatase and tensin homologue (PTEN), senescence biomarkers and TT-Cs in a formalin-fixed paraffin-embedded (FFPE) sample series of CAN and DN classified according to their conventional histopathological features and according to 2 different dermoscopic classifications.

MATERIALS AND METHODS

Acquired melanocytic naevi (volar excluded), excised from different patients, were identified prospectively, under clinical suspicion of CAN (junctional (JN), compound (CN), intradermal (IN)) or atypical naevi. Clinical and dermoscopic images were obtained. Two independent pathologists assessed the lesions and discussed discrepancies. Naevi were re-classified in order to obtain a final series of 80 naevi, diagnosed as 60 CAN (20 JN, 20 CN, 20 IN) and 20 DN (6).

Dermoscopic images were evaluated by 3 dermatologists without clinical or histopathological information. The naevi were classified according to global pattern (10) (applying a variation of pattern analysis) and to the 4 common patterns defined by Zalaudek et al. (11). Considered global patterns were reticular, globular/cobblestone, mixed reticulo-globular/reticulo-cobblestone (R-G/R-C) and multicomponent (MP). Zalaudek patterns were reticular, globular-cobblestone, mixed pattern with central globular or structureless brown area and peripheral network (CG/PR) and mixed pattern with central network or structureless brown area and peripheral globules (CR/PG). Discrepant cases were discussed.

FFPE tissue samples were subjected to immunohistochemistry (IHC) with antibodies against proliferation (Ki67), cell cycle (cyclin D1), oncogene induced (p16, p53, pRB), and replicative (telomerase: hTERT) senescence markers, PTEN, BRAF/V600E and 2 TT-C isoforms (Cav3.1, Cav3.2) (9). The optimal IHC conditions and procedures for each antibody are listed in Table SI[1]. Immunohistochemical staining was graded semiquantitatively by considering the percentage and intensity of the staining, resulting in a histoscore (*Hsc*) (ranges 0–300) (9).

Statistical analysis was carried out using GraphPad Prism software. Differential immunoexpression of biomarkers between histopathological and dermoscopic patterns were analysed by Kruskal–Wallis test with Dunn's Multiple Comparison test. *p*-values are indicated by asterisks *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

The study was approved by the local ethics committee (Comité Ètic d'Investigació Clínica de l'Hospital Arnau de Vilanova de Lleida. Study ID number: CEIC-1051) with a specific informed consent.

RESULTS

Clinical features of patients and naevi

Mean age of patients was 42.8 years, standard deviation (SD) 16.9 years; 73.7% were women and 26.3% men. Fifteen percent of naevi were located on the head and neck, 5% on the upper anterior trunk, 15% on the lower anterior trunk, 33.75% on the upper back, 8.75% on the lower back, 3.75% on the upper limbs, and 18.75% on the lower limbs. According to these locations, 15% of naevi were located on chronic sun exposure areas and 85% on intermittent sun exposure areas.

The distribution of DN and CAN according to age, sex and location was homogeneous.

The anatomical location of naevi, sex and age of the patients are shown in Table SII[1].

Dermoscopic characteristics of naevi

Global pattern. Twenty-five (31.25%) naevi had a reticular pattern, 23 (28.75%) globular/cobblestone, 17 (21.5%) mixed R-G/R-C and 15 (18.75%) MP (Fig. S1[1]). *Zalaudek's pattern*. Twenty-six (32.9%) naevi had a reticular pattern, 24 (30.4%) globular-cobblestone, 23 (29.1%) CG/PR and 6 (7.6%) CR/PG (Fig. S2[1]). One case was unclassifiable and was excluded.

No naevi with non-specific, homogeneous or starburst patterns were identified.

Correlation of dermoscopic and histopathological classifications

One-hundred percent of IN had a globular/cobblestone pattern. Approximately 40% of JN showed a reticular pattern, while CN often showed mixed patterns. Forty percent of DN were classified as MP in global pattern classification, whereas, in Zalaudek classification, DN were distributed among the remaining patterns (except for the globular/cobblestone) (Table SIII[1]).

Immunohistochemical evaluation of BRAF/V600E in the histological and dermoscopic groups

Mean *Hsc* for BRAF/V600E immunostaining was lower in DN than in CAN, with no statistical significance. Differences were significant ($p < 0.001$) when the 4 histopathological subtypes were compared, being especially
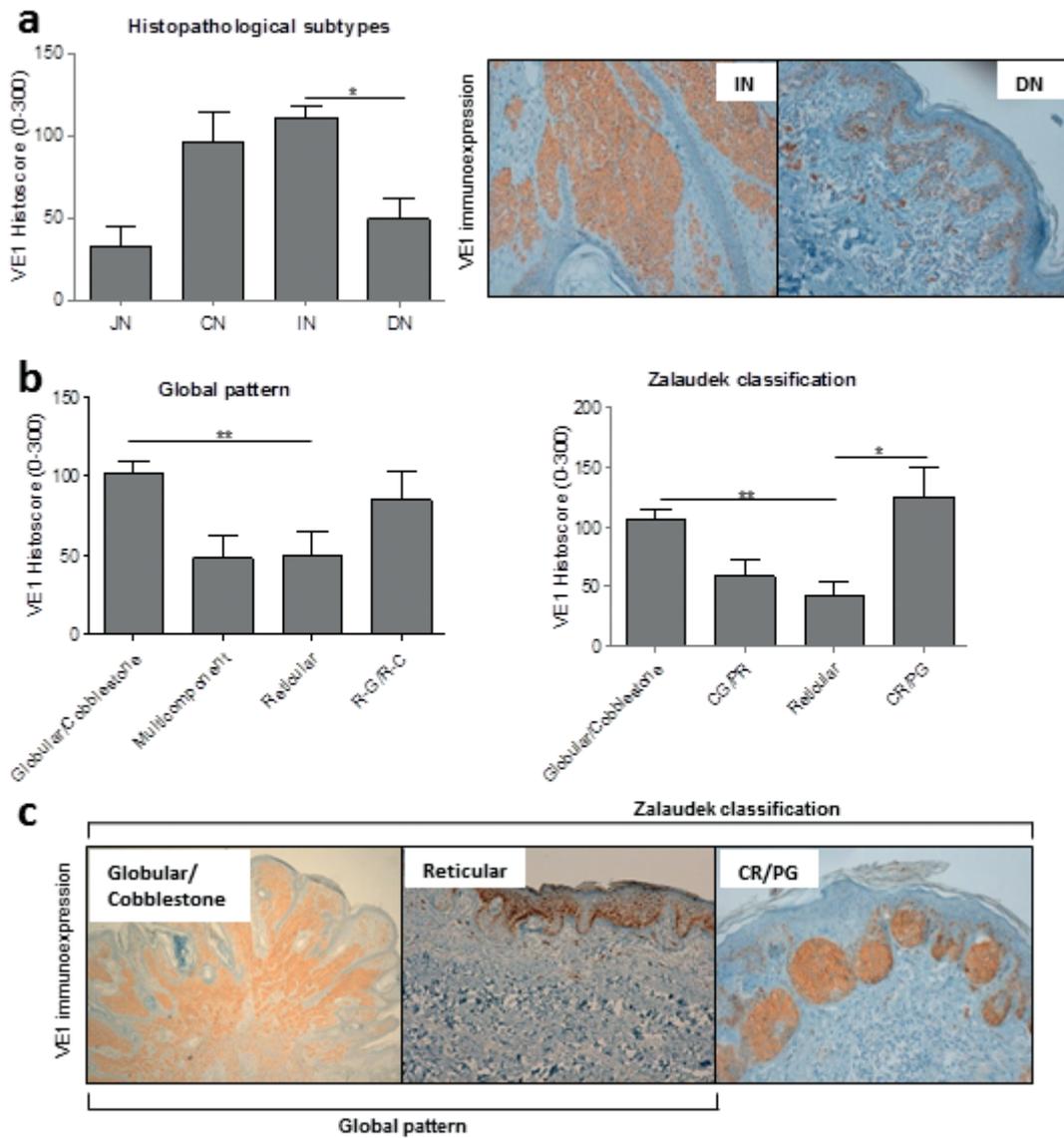
Fig. 1. Immunohistochemical evaluation of BRAF/V600E by VE1 antibody in different melanocytic naevi type according to histological and dermoscopic groups. (a) Positivity for BRAF/V600E was significantly lower in dysplastic naevi (DN) than in intradermal naevi (IN). (b) In both dermoscopic classifications, reticular pattern (in addition to multicomponent pattern of pattern analysis) showed the lowest BRAF/V600E expression. (c) Representative images for BRAF/V600E immunoexpression in different melanocytic naevi according to global pattern and Zalaudek classification. Statistical analysis was performed using Kruskal–Wallis test and Dunn's Multiple Comparison Test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; non-significant (n.s.)). JN: junctural naevi; CN: compound naevi; RG/RC: reticulo-globular/reticulo-cobblestone pattern; CG/PR: mixed pattern with central globular or structureless brown area and peripheral network; CR/PG: mixed pattern with central network or structureless brown area and peripheral globules.

Table I. Differential immunoexpression (*Hsc*) of biomarkers in the 4 different acquired naevus subtypes

		Histological naevi subtypes (4 subtypes)					Differences vs DN group		
Biomarker	Total n = 80 (100%) Mean (SD)	JN n = 20 (25%) Mean (SD)	CN n = 20 (25%) Mean (SD)	IN n = 20 (25%) Mean (SD)	DN n = 20 (25%) Mean (SD)	Global differences between the 4 groups	JN vs DN	CN vs DN	IN vs DN
Ki67	10.42 (10.75)	16.67 (8.16)	13.15 (9.05)	3.25 (4.67)	13 (13.9)	**<0.001**	n.s	n.s	**0.0009**
Cycl D1	97.2 (49.52)	100 (60.33)	110 (45.19)	93 (50.48)	87.75 (50.54)	0.47	–	–	–
p16	116.25 (75.48)	72 (64.2)	129.5 (61.94)	168 (69.91)	95.5 (72.94)	**<0.001**	n.s	n.s	**0.006**
PTEN	169.25 (62.64)	167 (68.6)	171.5 (50.29)	192.5 (42.16)	146 (78.43)	0.19	–	–	–
hTERT	188.75 (21.08)	198.5 (16.94)	183.5 (29.43)	167.5 (20.74)	205.5 (20.12)	**<0.001**	n.s	n.s	**<0.001**
VE1	72 (64.79)	33 (50.46)	95.5 (81.72)	110.5 (30.86)	49 (54.57)	**<0.001**	n.s	n.s	**0.002**
Cav3.1	17.75 (25.61)	11 (10.71)	18.5 (30.14)	3 (5.71)	38.5 (30.66)	**<0.001**	**0.001**	**0.007**	**<0.001**
Cav3.2	108.5 (46.83)	104 (48.28)	105 (58.26)	117 (30.11)	108 (48.73)	0.93	–	–	–

Significance of the differences was evaluated with non-parametric tests (Mann–Whitney test or Kruskal–Wallis test, whichever was most convenient).
SD: standard deviation; JN: junctural naevi; CN: compound naevi; IN intradermal naevi; DN: dysplastic naevi; PTEN: phosphatase and tensin homologue; VE1: BRAF/V600E; n.s: not significant; hTERT: telomerase; cycl D1: cycline D1. Statistically significant differences are highlighted in bold numbers.

Table II. Differential immunoexpression (*Hsc*) of biomarkers according to dermoscopic global patterns

Biomarker	Total $n=80$ (100%) Mean (SD)	Global patterns				Global differences between the 4 groups
		Globular/cobblestone $n=23$ (28.75%) Mean (SD)	Multicomponent $n=15$ (18.75%) Mean (SD)	Reticular $n=25$ (31.25%) Mean (SD)	R-G/R-C $n=17$ (21.25%) Mean (SD)	
Ki67	10.42 (10.75)	4.39 (5.71)	13.46 (12.14)	15 (12.62)	12.08 (9.72)	**0.001**
Cycl D1	97.2 (49.52)	112.94 (48.19)	95.38 (61.46)	95.88 (41.54)	98.08 (48.03)	0.23
p16	116.25 (75.48)	163.9 (70.93)	82 (61.09)	84.4 (65.13)	128.82 (73.9)	**0.001**
PTEN	169.25 (62.64)	192.6 (39.34)	124 (84.33)	166.8 (52.66)	181.2 (63.23)	**0.02**
hTERT	188.75 (21.08)	164.7 (22.39)	190.7 (19.07)	201.6 (23.40)	198.8 (23.95)	**<0.001**
VE1	72 (64.79)	101.7 (38.33)	48 (55.83)	50.2 (73.18)	85 (72.2)	**0.008**
Cav3.1	17.75 (25.61)	3.478 (6.473)	27.33 (28.9)	25.6 (30.29)	17.06 (24.43)	**<0.001**
Cav3.2	108.5 (46.83)	121.76 (37.79)	102.67 (56.5)	109.6 (37.8)	101.18 (61.73)	0.78

Significance of the differences was evaluated with non-parametric tests (Kruskal–Wallis test).
SD: standard deviation; R-G/R-E: reticulo-globular/reticulo-cobblestone pattern; PTEN: phosphatase and tensin homologue; VE1: BRAF/V600E; hTERT: telomerase; cycl D1: cycline D1. Statistically significant differences are highlighted in bold numbers.

high in IN, medium in CN, low in DN and even lower in JN. However, if each group of CAN was compared with DN, statistical significance was only found regarding DN vs IN ($p=0.002$) (**Fig. 1**, **Table I** and Table SIV[1]).

In both dermoscopic classifications (global pattern and Zalaudek's), globular/cobblestone pattern showed a high BRAF/V600E *Hsc* and reticular pattern a low *Hsc*. However, in global pattern classification, MP showed the lowest value and, in Zalaudek classification, CR/PG pattern showed the highest value. Compared in pairs, only differences related to reticular vs globular/cobblestone naevi ($p<0.01$) remained significant for global pattern classification (Fig. 1 and **Table II**) and reticular vs globular/cobblestone ($p<0.05$) and reticular vs CR/PG naevi ($p<0.01$) for Zalaudek classification (Fig. 1 and **Table III**).

Differential immunoexpression of the remaining biomarkers in histopathological and dermoscopic subtypes

Regarding other biomarkers, if CAN vs DN were compared, significant differences were only seen regarding hTERT and Cav.3.1 immunoexpression, which were higher in DN ($p<0.001$ for both biomarkers). Expression of p16 and PTEN was lower in DN, but not significant.

Immunoexpression of pRB and p53 was extremely low and was considered negative (Table SIV[1]).

If each CAN subgroup was compared with DN, DN showed significantly higher Ki67 and hTERT *Hsc* ($p=0.0009$ and $p=0.006$, respectively) and lower p16 *Hsc* ($p<0.001$) than IN and higher Cav3.1 *Hsc* than any of the 3 CAN subtypes ($p=0.001$ vs JN, $p=0.007$ vs CN and <0.001 vs IN) (Table I).

In both dermoscopic classifications, globular/cobblestone pattern showed significantly high levels of p16 and low levels of Ki67, hTERT and Cav3.1. According to global pattern, immunoexpression of both p16 and PTEN was significantly low in MP ($p<0.05$ for both markers vs globular/cobblestone naevi), which also showed the highest levels of Cav3.1 ($p<0,01$ vs globular/cobblestone naevi). No differences in PTEN immunoexpression were detected when Zalaudek classification was applied. Zalaudek's CR/PG pattern showed the highest levels of hTERT and Cav3.1, significant vs globular/cobblestone pattern ($p<0.05$ and $p<0.01$ respectively). Although CR/PG naevi showed the highest Ki67 *Hsc*, differences were not significant in the multiple comparisons (**Figs 2** and **3** and Tables II and III).

No differences in Cav3.2 or in cyclin D1 immunoexpression were seen in any of the comparisons.

Table III. Differential immunoexpression of biomarkers according to dermoscopic Zalaudek patterns

Biomarker	Total $n=79$ (100%) Mean (SD)	Zalaudek patterns				Global differences between the 4 groups
		Globular/cobblestone $n=24$ (30.38%) Mean (SD)	CG/PR $n=23$ (29.11%) Mean (SD)	Reticular $n=26$ (32.91%) Mean (SD)	CR/PG $n=6$ (7.59%) Mean (SD)	
Ki67	10.43 (10.84)	5.88 (9.17)	12.94 (11.05)	13.16 (11.93)	13.4 (8.38)	**0.006**
Cycl D1	97.62 (49.79)	101.3 (52.03)	104.1 (47.44)	77.63 (43.35)	134 (53.2)	0.14
p16	116.58 (75.9)	160.4 (71.46)	105.2 (81.62)	86.92 (64.73)	113.33 (41.31)	**0.011**
PTEN	170.63 (61.8)	192.1 (38.56)	152.6 (76.23)	158.8 (58.4)	205 (65.35)	0.11
hTERT	188.75 (21.08)	165.8 (21.04)	194.8 (22.74)	201.5 (22.92)	201.7 (17.22)	**<0.001**
VE1	72.91 (64.69)	106.3 (43.52)	58.91 (63.64)	42.50 (64.02)	125 (62.85)	**<0.001**
Cav3.1	17.59 (25.73)	4.17 (7.17)	14.78 (22.54)	27.69 (31.54)	38.33 (30.61)	**<0.001**
Cav3.2	119.62 (46.03)	117.5 (36.62)	90.87 (58.54)	111.9 (38.89)	140 (34.64)	0.12

Significance of the differences was evaluated with non-parametric tests (Kruskal–Wallis test).
SD: standard deviation; CG/PR: mixed pattern with central globular or brown area without structure and peripheral network, CR/PG: mixed pattern with central network or brown area without structure and peripheral globules; PTEN: phosphatase and tensin homologue; VE1: BRAF/V600E; hTERT: telomerase; cycl D1: cyclin D1. Statistically significant differences are highlighted in bold numbers.
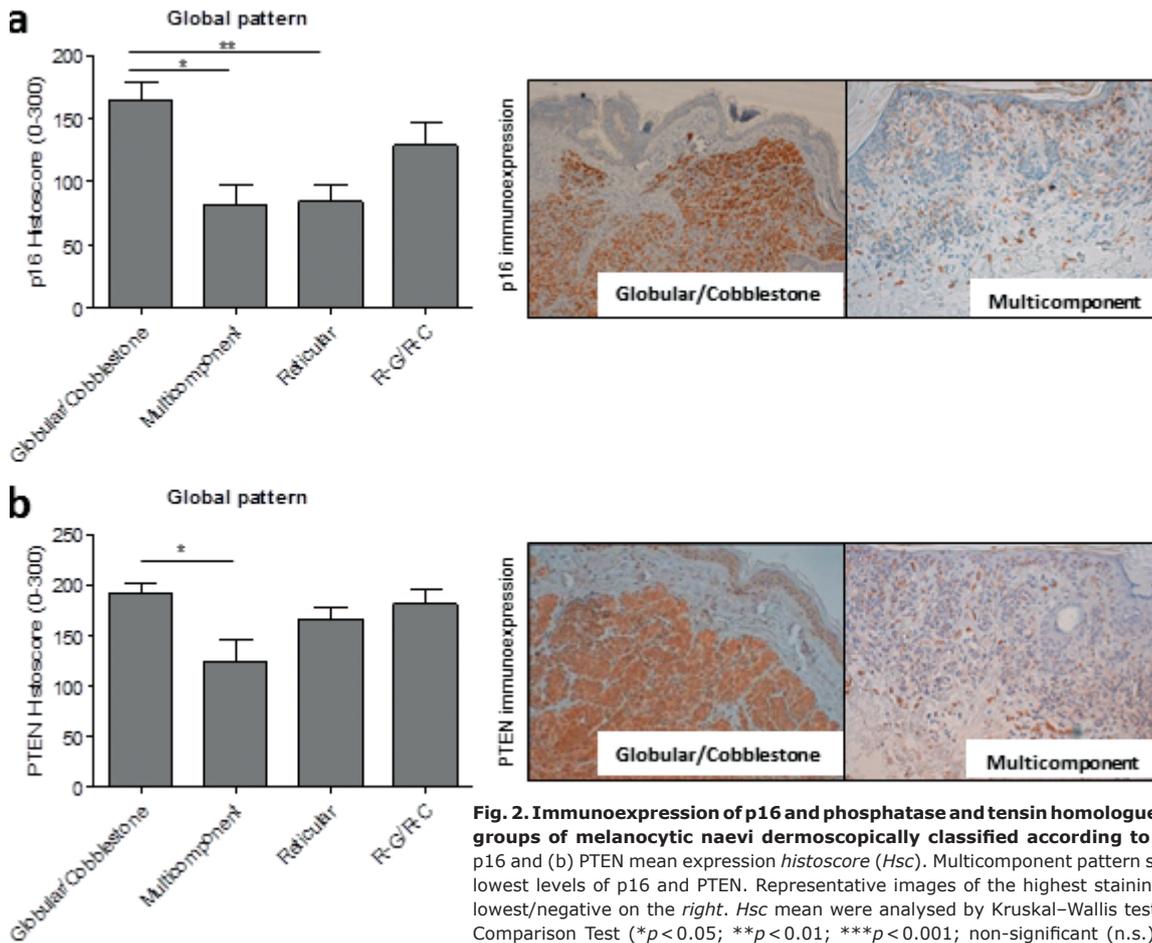
Fig. 2. Immunoexpression of p16 and phosphatase and tensin homologue (PTEN) in different groups of melanocytic naevi dermoscopically classified according to global pattern. (a) p16 and (b) PTEN mean expression *histoscore* (*Hsc*). Multicomponent pattern showed the significant lowest levels of p16 and PTEN. Representative images of the highest staining on the *left* and the lowest/negative on the *right*. *Hsc* mean were analysed by Kruskal–Wallis test and Dunn's Multiple Comparison Test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; non-significant (n.s.)). R-G/R-C: reticulo-globular/reticulo-cobblestone pattern.

DISCUSSION

This study found that CAN expressed higher levels of V600E than DN. IN showed the highest levels, followed by CN, DN and JN, which is in agreement with other published data (2).

The results regarding BRAF-IHC and dermoscopic classification are also in agreement with the literature (8, 9). *BRAF* mutations have been described in 92% of globular and 100% of CR/PG naevi, whereas reticular naevi were 67% *BRAF*- and 33% *NRAS*-mutant (8). In the current study, naevi with the lowest *Hsc* displayed a reticular pattern (which included JN, CN and DN), whereas globular/cobblestone naevi (most IN) presented a high *Hsc*. The *Hsc* of the growing CR/PG pattern, which does not have a translation in the histopathological classification (DN, CN and JN in this series) was even higher.

Regarding other biomarkers, Ki67 and hTERT expression were significantly higher and p16 significantly lower in DN vs IN. Previous studies indicated an increase in proliferation markers and p16 losses in DN and intermediate melanocytic lesions (3, 5) and a greater immunoexpression of hTERT in DN than CAN (12) or, more specifically, in DN vs IN (13). Moreover, *TERT* promoter mutations were described as early alterations in intermediate lesions (5). These findings are all in agreement with the results of the current study. Other studies have shown discrepancies regarding the presence of TERT promoter mutations in melanocytic naevi (4), probably due to technical issues (14). Moreover, since the current study is IHC based, more proliferative naevus subtypes in comparison with IN may show increased expression of hTERT in the absence of *hTERT* alterations.

Dermoscopically atypical MP naevi (DN, JN and CN in the current study), expressed high Ki67 and significantly low p16 levels (like DN) but also significantly low PTEN immunoexpression (unlike DN). PTEN is a tumour suppressor whose expression has been described as high in naevus and low in melanoma (15), but there is no published information about PTEN expression in CAN vs DN. PTEN mutations and promoter methylation appear to be exclusive of melanoma (5), with the exception of xeroderma pigmentosum atypical naevi (16). Nevertheless, the current results, with a validated PTEN immunostaining method (17), showed especially low levels in MP. More recently, p16 and PTEN and other gene copy number aberrations have been described in benign naevi, especially with reticular vs globular pattern (4). Although in that study more specific patterns,
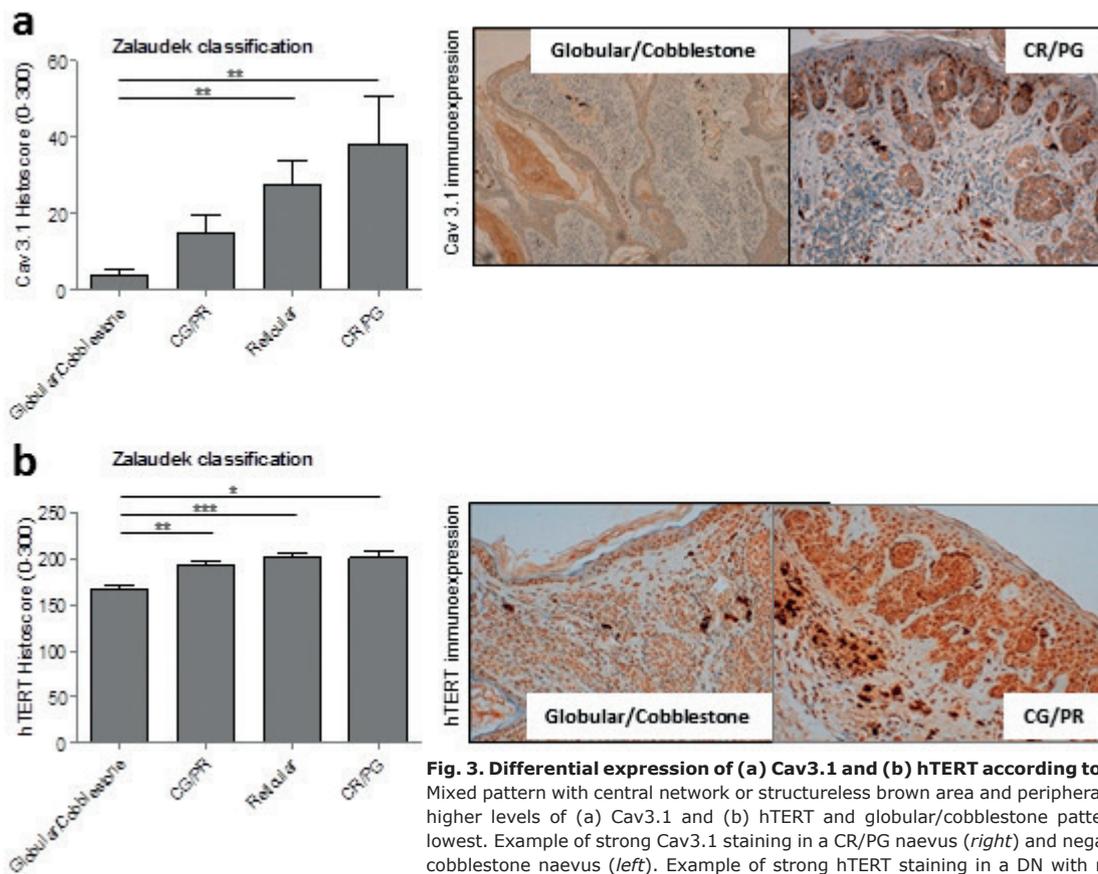
Fig. 3. Differential expression of (a) Cav3.1 and (b) hTERT according to Zaudek classification. Mixed pattern with central network or structureless brown area and peripheral globules (CR/PG) showed higher levels of (a) Cav3.1 and (b) hTERT and globular/cobblestone pattern showed the significant lowest. Example of strong Cav3.1 staining in a CR/PG naevus (*right*) and negative staining in a globular/cobblestone naevus (*left*). Example of strong hTERT staining in a DN with mixed pattern with central globular or structureless brown area and peripheral network (CG/PR) (*right*) and lower staining in a globular/cobblestone lesion (*left*). *Histoscore* (*Hsc*) mean were analysed by Kruskal–Wallis test and Dunn's Multiple Comparison Test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; non-significant (n.s.)).

such as MP or mixed patterns, were not considered, this finding could explain the current results.

Zalaudek CR/PG pattern was associated with higher IHC levels of hTERT, and also high, although not statistically significant, levels of Ki67, as expected for growing naevi.

As for TT-Cs, the current results were coherent with our published findings (6). We did not observe significant differences in the distribution of Cav3.2 (6), while DN showed a significantly higher *Hsc* of Cav3.1 compared with the whole group of CAN (as described by our group) (6), but also to each individual histological CAN subtype (JN, CN and IN), something that was not previously compared. Moreover, the highest Cav3.1 *Hsc* were associated with MP and with the growing CR/PG pattern.

Study limitations

This study has some limitations. It was designed to have a well-balanced sample regarding histopathological subtypes of acquired naevi, but not regarding age, sex and anatomical location (also not regarding dermoscopic subtypes). The final sample contained more women than men, and naevi were removed from different body sites in different patients. This may have an impact, as it is well known that there are individual dermoscopy signatures which are characteristic for the individual patient. Finally, when the naevi were re-classified according to dermoscopic criteria, some of the groups were relatively small. Hence, it is necessary to confirm these findings in a larger series of naevi in order to cover the specific aspects mentioned.

Conclusion

In summary, DN showed lower BRAF/V600E immunoexpression than CAN. This was significant in comparison with IN, which showed the highest BRAF/V600E *Hsc*. Naevi with the lowest BRAF/V600E levels were JN. Globular/cobblestone and reticular dermoscopic patterns were consistently associated with high and low BRAF/V600E *Hsc*, respectively, but CR/PG pattern of Zalaudek's classification (which does not have a translation in conventional histopathology classification and is not considered in global pattern analysis) showed the highest *Hsc*. Among global patterns the previously not analysed MP exhibited the lowest levels of BRAF/V600E. Moreover, the atypical MP naevi presented low p16 and PTEN *Hsc* and the growing CR/PG naevi high Ki67, hTERT and Cav3.1 levels. In conclusion, histopathology and dermoscopy provide complementary

information about the biological behaviour of melanocytic naevi.

ACKNOWLEDGEMENTS

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