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BRAF, *NRAS* and *HRAS* mutations in spitzoid tumours and their possible pathogenetic significance

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Conflicts of interest

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Summary

Background The relationships between so-called spitzoid tumours have proven difficult to understand.

Objectives To address three questions: does spitzoid tumour morphological similarity reflect molecular similarity? Does Spitz naevus progress into spitzoid melanoma? Are ambiguous spitzoid tumours genuine entities?

Methods *BRAF*, *NRAS* and *HRAS* mutations were analysed using single-strand conformational polymorphism analysis and sequencing.

Results Both Spitz naevi and spitzoid melanoma had a lower combined *BRAF* and *NRAS* mutation frequency compared with common acquired naevi ($P = 0{\cdot}0001$) and common forms of melanoma ($P = 0{\cdot}0072$), respectively. To look for evidence of progression from Spitz naevi to spitzoid melanoma, *HRAS* was analysed in 21 spitzoid melanomas, with no mutations identified. The binomial probability of this was 0·03 based on an assumption of a 15% mutation frequency in Spitz naevi with unbiased progression. Under these assumptions, *HRAS* mutations must be rare/absent in spitzoid melanoma. Thus, Spitz naevi seem unlikely to progress into spitzoid melanoma, implying that ambiguous spitzoid tumours cannot be intermediate degrees of progression. In addition, the data suggest that *HRAS* mutation is a potential marker of benign behaviour, in support of which none of three *HRAS* mutant spitzoid cases metastasized.

Conclusions First, the morphological similarity of spitzoid tumours reflects an underlying molecular similarity, namely a relative lack of dependence on *BRAF*/*NRAS* mutations. Second, Spitz naevi do not appear to progress into spitzoid melanoma, and consequently ambiguous spitzoid tumours are likely to be unclassifiable Spitz naevi or spitzoid melanoma rather than genuine entities. Third, *HRAS* mutation may be a marker of Spitz naevus, raising the possibility that other molecular markers for discriminating Spitz naevi from spitzoid melanoma can be discovered.

The Spitz naevus is a benign usually acquired melanocytic tumour that is characteristically composed of spindle and/or epithelioid naevomelanocytes. It was originally described in 1948 as 'juvenile melanoma',[1] this name reflecting the fact that Spitz naevi have several features in common with melanoma. Indeed, those melanomas showing most similarity to Spitz naevi are commonly referred to as spitzoid melanoma. Because Spitz naevi and spitzoid melanoma (generically referred to as spitzoid tumours in this paper) have several overlapping features, there is a minority of spitzoid tumours that cannot readily be classified.[2,3] These ambiguous spitzoid tumours have been given various names such as atypical Spitz tumour and spitzoid tumour of uncertain malignant potential (STUMP). The relationship of these lesions to each other is the subject of a debate[4,5] from which three key questions can be distilled. First, does the morphological similarity within the spitzoid tumour family reflect a molecular similarity? Second, does Spitz naevus progress into spitzoid melanoma? Third, are ambiguous spitzoid tumours genuine entities?

With regard to the first question, histology provides a precedent because there are several groups of benign and malignant melanocytic tumours that seem to be related through common clinicopathological features.[6] For example, common acquired naevi and melanomas from intermittently

sun-exposed skin seem to form a group, as shown by such naevi occasionally progressing to melanoma, and also by molecular analysis showing that these same naevi and melanomas have a high frequency of *BRAF* mutations,[7] in keeping with a common molecular mechanism. So, in principle, there is no reason why spitzoid tumours could not also form a related group of melanocytic tumours. Indeed, molecular analysis suggests that spitzoid tumours may be distinct from common acquired naevi and melanomas from intermittently sun-damaged skin because spitzoid tumours have rare *BRAF* and *NRAS* mutations.[8,9] However, there is some dispute about the frequency of *BRAF* and *NRAS* mutation in spitzoid tumours, as others have found mutations to be more frequent.[10–12]

With regard to the second question, it is unknown whether Spitz naevus has the capability to progress into spitzoid melanoma. There are no unequivocal clinical reports of this phenomenon and neither does molecular analysis provide any supportive evidence that such progression occurs.[13] If it does occur, then molecular changes that are present in Spitz naevi should be found in spitzoid melanoma. Unfortunately, the molecular basis of Spitz naevi is largely unknown. Nevertheless, one molecular change, 11p gain, has been found in over 20% of Spitz naevi.[14] The putative target of this gain is the *HRAS* locus, and in approximately two thirds of these cases the gene harbours an activating mutation,[15] with a mutation frequency of up to 29% in Spitz naevi.[12] However, so far there have been no reports of *HRAS* mutation in spitzoid melanoma, suggesting that Spitz naevus does not progress to spitzoid melanoma, but this must be balanced against the fact that there have been relatively few studies done.[12,16]

Thirdly, the status of ambiguous spitzoid lesions is especially contentious. In particular, it is unclear whether they are genuine intermediate lesions that lie in a progression pathway between Spitz naevus and a spitzoid melanoma, or whether they are merely unclassifiable lesions that, with better criteria, could be correctly diagnosed as Spitz naevus or spitzoid melanoma. The answer to this question is intimately tied into whether Spitz naevi progress to spitzoid melanoma. If such progression occurs, then ambiguous spitzoid tumours may well represent *bona fide* entities that are part of that continuum. If not, then ambiguous spitzoid tumours are unlikely to be genuine entities. Understanding the nature of ambiguous spitzoid tumours therefore requires knowledge of whether there is evidence of progression between Spitz naevus and spitzoid melanoma.

With these issues in mind, the aims of the present study were first to assess whether spitzoid melanoma is different from nonspitzoid melanoma by looking at the combined frequency of *BRAF* and *NRAS* mutation (the combined frequency was analysed because these two alterations appear to be reciprocal mechanisms of MAP kinase pathway alteration). The second aim was to assess whether Spitz naevi progress to spitzoid melanoma by analysing *HRAS* mutation, which in turn would shed light on the third question regarding the nature of ambiguous spitzoid tumours.

Materials and methods

Cases

Initially, 90 lesions classified as Spitz naevus (n = 17), nonclassical spitzoid tumours (including ambiguous spitzoid tumours and spitzoid melanoma) (n = 38) and nonspitzoid melanoma (n = 35) were obtained. Spitz naevi and nonspitzoid melanomas were obtained solely from the tissue archives of Leicester Royal Infirmary from a search of the pathology database spanning the years 1987–2005 for Spitz naevi and 1987–2004 for melanomas. To be eligible for inclusion the final diagnosis of the histology report had to be unequivocal. Spitz naevi and nonspitzoid melanomas that were deemed eligible were chosen sequentially from the database allowing for matching as closely as possible of sex, age and site with the third group of tumours, nonclassical spitzoid tumours. These, being rarer, were obtained both locally and from collaborating histopathology departments in the U.K. and U.S.A., and were included if the histology report made reference to the lesion having a spitzoid morphology and detailed some uncertainty concerning the malignant potential of the lesion. The spitzoid lesions were from institutes across the U.K. and U.S.A. and so the diagnostic categories to which these challenging lesions were assigned lacked consistency. Therefore, to achieve consistency in terminology and avoid inevitable disagreements that would arise from independent review, two dermatopathologists (A.F. and M.B.) reviewed the cases together at a double-headed microscope and reached a consensus opinion by open discussion of each case in order to assign them to one of four spitzoid tumour categories (see below). The pathologists were blinded to the initial diagnosis, the centre from which the case came, the clinical data (age, sex and site), the clinical outcome and the results of mutation analysis. They reviewed all 90 cases and assigned the lesions to a diagnostic category. There is no universally accepted definition of spitzoid morphology but the following criteria were applied based upon frequently described features of spitzoid lesions: the overwhelming majority of cells have a spindle and/or epithelioid morphology with characteristic large nuclei containing prominent and brightly eosinophilic nucleoli and abundant eosinophilic cytoplasm alongside a constellation of features deemed useful for discriminating Spitz naevus from spitzoid melanoma, namely lesional size, symmetry, circumscription, ulceration, pagetoid spread, pleomorphism, mitotic count, mitotic location, maturation, depth and subcuticular involvement;[1,17] additionally, to be classified as spitzoid, the junctional features did not have a radial growth phase consistent with superficial spreading, acral lentiginous or lentigo maligna melanoma. Cases that were not considered to meet the criteria for 'spitzoid' were either diagnosed as nonspitzoid melanomas (n = 25) or discarded (n = 0). The cases with spitzoid morphology were ultimately assigned to one of the following four categories by the reviewing pathologists: Spitz naevus (n = 16), atypical Spitz naevus (cases with slightly atypical histological features but likely to be Spitz naevus, n = 9),

Table 1 Clinicopathological data for the tumour series

Clinical and pathological parameters	Spitz naevi (n = 16)	Atypical Spitz naevi (n = 9)	STUMP (n = 9)	Spitzoid melanoma (n = 27)	Nonspitzoid melanoma (n = 25)
Sex					
Male	8 (50%)	5 (56%)	2 (22%)	13 (48%)	11 (44%)
Female	8 (50%)	4 (44%)	6 (67%)	14 (52%)	14 (56%)
Missing data	–	–	1 (11%)	–	–
Age at diagnosis (years), mean ± SD	30·3 ± 13·3	35·4 ± 15·6	31·7 ± 11·2	31·3 ± 22·8	37·3 ± 24·6
Tumour site					
Head and neck	2 (12%)	2 (22%)	–	6 (22%)	5 (20%)
Trunk	1 (6%)	–	2 (22%)	5 (18%)	3 (12%)
Upper extremity	1 (6%)	2 (22%)	2 (22%)	5 (18%)	4 (16%)
Lower extremity	10 (62%)	4 (44%)	4 (44%)	9 (33%)	12 (48%)
Missing data	2 (12%)	1 (11%)	1 (11%)	2 (7%)	1 (4%)
Tumour thickness (mm), mean ± SD	1·8 ± 1·2	2·0 ± 1·7	4·2 ± 3·4	3·2 ± 2·9	2·5 ± 2·6
Outcome					
Disease free	16 (100%)	8 (89%)	7 (78%)	21 (78%)	22 (88%)
Recurrence or metastasis	–	1 (11%)	2 (22%)	6 (22%)	3 (12%)
Follow up period (years), mean ± SD	9·5 ± 6·2	8·2 ± 5·3	5·7 ± 3·2	9·8 ± 6·9	7·1 ± 4·2

STUMP, spitzoid tumour of uncertain malignant potential.

STUMP (cases with more marked histological deviation from classical Spitz naevus, n = 9) and spitzoid melanoma (spitzoid features, but frankly malignant, n = 27). Four cases were discarded because there was insufficient tissue. The clinicopathological features of the cases are shown in Table 1 while supplementary Table S1 (see Supporting information) includes details for each individual case. Twelve of the cases were metastatic: one of nine atypical Spitz naevi (diagnosed by sentinel node biopsy), two of nine STUMP, six of 27 spitzoid melanoma (two diagnosed by sentinel node biopsy) and three of 25 nonspitzoid melanoma. Research Ethics Committee approval was obtained for this project.

Mutation analysis

Mutations in *BRAF*, *NRAS* and *HRAS* genes were detected using single-strand conformational polymorphism (SSCP) analysis of polymerase chain reaction (PCR) amplification of DNA extracted from one to five 10-μm tissue sections per sample. Enrichment of DNA from melanoma cells was performed by removing the tumour tissue from 10-μm sections with a pipette tip. The tumour tissue was identified by comparison with a haematoxylin and eosin-stained serial section. Microdissection, DNA extraction, SSCP analysis and sequencing were performed as described previously.[18] The primer sequences used to amplify the target exons for *BRAF* exon 15, *NRAS* exons 2 and 3 and *HRAS* exons 2 and 3 are shown in Table 2.

Statistical analysis

Mutation frequencies were compared using Fisher's exact test. For metastasis-free survival, time to metastasis was calculated from the date of primary melanoma diagnosis to the date of event, with patients censored at last follow up. Survival curves were generated according to the Kaplan–Meier product-limit method and were compared using the log-rank test. All tests

Table 2 Primer sequences for polymerase chain reaction amplification of the exons of interest in *BRAF*, *NRAS* and *HRAS* genes

Gene	Exon	Forward primer position	Forward primer sequence	Reverse primer position	Reverse primer sequence
BRAF	15	1044769 : 1044748	TTTCCTTTACTTACTACACCTC	1044581 : 1044601	TCAGGGCCAAAAATTTAATCA
NRAS	2	11166518 : 11166499	CTCGCCAATTAACCCTGATT	11166306 : 11166325	CCGACAAGTGAGAGACAGGA
	3	11164293 : 11164274	CACCCCCAGGATTCTTACAG	11164169 : 11164188	TCGCCTGTCCTCATGTATTG
HRAS	2	474368 : 474350	AGGAGACCCTGTAGGAGGA	474200 : 474221	CGCTAGGCTCACCTCTATAGTG
	3	474013 : 473994	AGAGGCTGGCTGTGTGAACT	473753 : 473772	TCACGGGGTTCACCTGTACT

Primer postions are located in the following reference sequences: *BRAF* NT_007914.14: *NRAS* NT_019273.18: *HRAS* NT_035113.6.

were two tailed and $P \leq 0{\cdot}05$ was considered statistically significant. Statistical analyses were performed using Statistical Package for the Social Sciences release 12.0 (SPSS, Chicago, IL, U.S.A.). Equivalence testing was performed as described,[19] using an arbitrary tolerance of > 10% difference in mutation frequency as nontrivial.

Results

BRAF and *NRAS* mutation frequency in spitzoid tumours

In order to assess whether the shared morphological features of spitzoid tumours reflect a common underlying molecular pathology, *BRAF* and *NRAS* mutations were analysed as these are frequently altered in many melanocytic tumours. The combined *BRAF* and *NRAS* mutation frequency in all spitzoid tumours, where complete analysis could be obtained, was 10 of 55 (18%). The mutations comprised one of 14 (7%) Spitz naevi, one of eight (13%) atypical Spitz naevi, three of eight (38%) STUMP and five of 25 (20%) spitzoid melanomas. The missing data in this and subsequent analyses were due either to lack of tissue or to PCR failure. Full mutation data for each case are given in supplementary Table S1 (see Supporting information). The analysis for this part of the study was directed towards unequivocally benign Spitz naevi and unequivocally malignant spitzoid melanoma because the status of ambiguous spitzoid tumours and STUMP as distinct entities remains uncertain (an issue dealt with in a separate analysis below). No statistically significant difference in mutation frequencies between spitzoid melanomas and Spitz naevi in patients of similar age and gender was detected ($P = 0{\cdot}39$). However, this does not prove equivalence; therefore a statistical test of equivalence was performed. This failed to reject the possibility of an important difference in mutation frequency (see Fig. 1).

BRAF and *NRAS* mutation frequency in spitzoid tumours and other melanocytic tumours

An assessment was made of whether the combined *BRAF* and *NRAS* mutation frequency in spitzoid tumours was different from that in other more common types of melanocytic tumour. We have previously analysed common acquired naevi, where 15 of 16 had a *BRAF* or *NRAS* mutation.[18] This frequency was significantly different from the frequency in Spitz naevi in the present study ($P = 0{\cdot}0001$). The mutation frequency in spitzoid melanoma was compared with that in more common forms of melanoma that were selected in a biased manner to include the same mix with respect to age, sex and site as the spitzoid melanoma group. The combined *BRAF* and *NRAS* mutation frequency in nonspitzoid melanoma was 14 of 23 (61%), three of which had regional node metastasis. In spitzoid melanoma, the frequency was five of 25 (20%), two of these having in-transit or regional node metastasis. The mutation frequency was significantly different ($P = 0{\cdot}0072$). Representative examples of mutation analysis are shown in Figure 2. These data suggest that while an equivalent combined *BRAF* and *NRAS* mutation frequency in Spitz naevus and spitzoid melanoma cannot be established, these lesions as a group still have a low mutation frequency, and so are much more likely to arise via *BRAF*/*NRAS*-independent mechanisms compared with the commonest forms of naevus and melanoma. The overall *BRAF* and *NRAS* mutation frequency in all spitzoid tumours, where complete analysis could be obtained, was also significantly different from that in common melanoma ($P = 0{\cdot}001$).

BRAF and *NRAS* mutation and spitzoid tumour metastasis

Given that *BRAF* and *NRAS* mutations are relatively common in nonspitzoid melanoma but not in spitzoid tumours, it is possible that these mutations might be indicative of metastatic potential in a spitzoid lesion. Among the spitzoid tumours, five of 45 (11%) *BRAF* and *NRAS* wild-type cases showed evidence of metastasis, with median follow up 6·5 years (interquartile range 3·8–12·6), compared with three of 10 (30%) mutant cases, with median follow up 6·2 years (interquartile range 5·3–13·3). This difference was not statistically significant ($P = 0{\cdot}15$). A log-rank test showed no significant difference in metastasis-free survival ($P = 0{\cdot}31$), although with only eight metastatic events among those cases where *BRAF*/*NRAS* mutation assessment was possible, this analysis was of limited value.

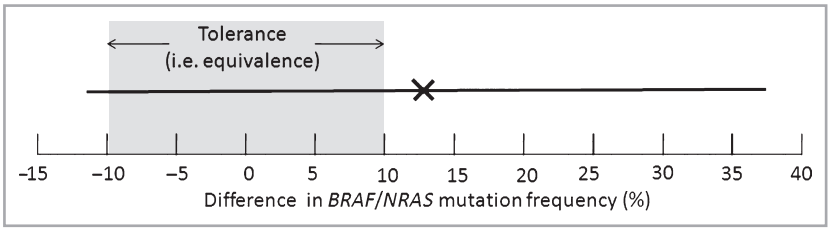

Fig 1. Equivalence test of mutation frequency in Spitz naevus and spitzoid melanoma. A tolerance was specified, representing a difference in combined *BRAF* and *NRAS* mutation frequency that was considered trivial. This was arbitrarily set at 10%. The actual difference and its 95% confidence interval (CI) was plotted (represented by the cross and the horizontal bold line, respectively). If the 95% CI is entirely within the tolerance zone, this is strong evidence that mutation frequencies are equivalent. If the 95% CI is entirely outside the tolerance zone, this is strong evidence on nonequivalence. If the 95% CI is only partially in the tolerance zone, as in the present data, then the result is ambiguous.

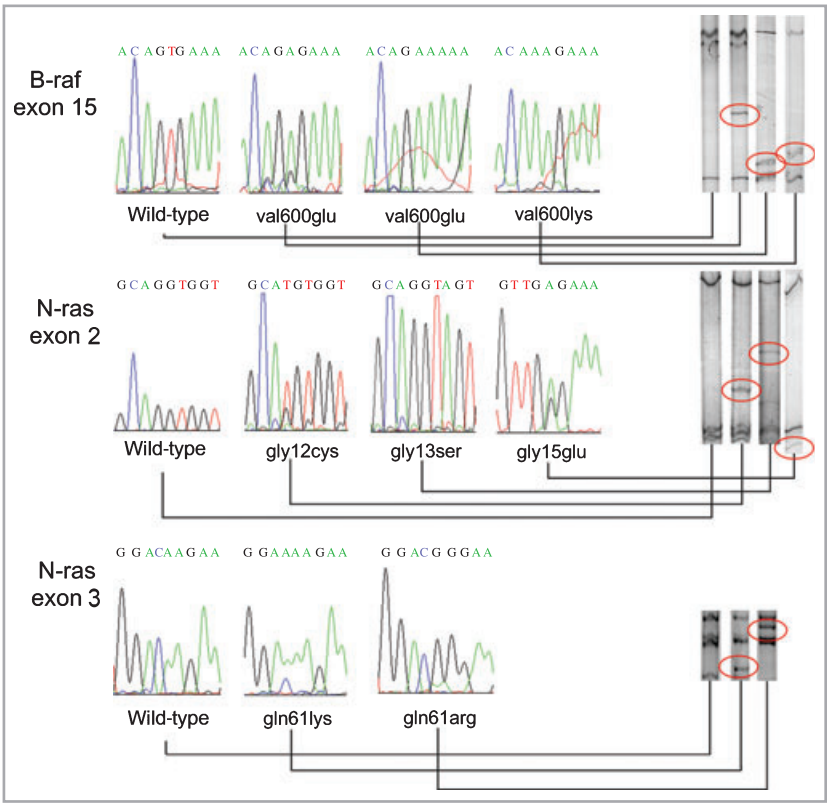

Fig 2. Single-strand conformational polymorphism analysis with accompanying sequencing electropherograms from representative cases with wild-type and mutant *BRAF* exon 15, *NRAS* exon 2 and *NRAS* exon 3. Aberrantly migrating bands are encircled in red.

BRAF mutation, *NRAS* mutation and age in spitzoid tumours

Some authors have suggested that age may influence the development of *BRAF* and *NRAS* mutations in spitzoid melanoma.[12] In the current study, there was no significant difference between the mean ± SD age of patients with *BRAF* and/or *NRAS* mutant spitzoid melanomas (29·0 ± 27·7 years, n = 5), and those with wild-type tumours (32·7 ± 22·0 years, n = 20) (P = 0·68). Furthermore, when the analysis included all spitzoid tumours, the mean ± SD age of *BRAF* and/or *NRAS* mutant patients (32·3 ± 19·4 years, n = 10) did not differ significantly from those with wild-type tumours (30·7 ± 17·7 years, n = 45) (P = 0·77). Next, patients were grouped according to whether they were greater or less than 10 years of age, to examine whether tumour mutations were different between pre- and postpubertal patients. The cut-off of ≤ 10 years of age has been utilized by other authors to define these two groups.[20] The biology of melanoma in children is poorly understood and it has been suggested by some authors that melanoma in children may behave differently from that in adults.[21–23] There were four patients aged < 10 years with spitzoid melanomas, of whom one (25%) harboured a *BRAF* mutation. This mutation was in a tumour removed from the buttock of a 5-year-old girl who was disease free after 5·3 years follow up. There were 21 spitzoid melanomas in patients over 10 years of age, four of which harboured *BRAF* or *NRAS* mutations (19%). There was no significant difference in the proportion of mutant cases between the two groups (P = 1), although the small number of cases in the group under 10 years of age precludes definitive comment. Similarly, the proportion of mutant cases varied little between the two groups when the analysis was expanded to include all spitzoid lesions, with one of six (17%) mutants in patients ≤ 10 years of age and nine of 49 (18%) in those over 10 years (P = 1). Because the number of patients aged 10 years or less was small, an alternative cut-off of 20 years of age or less was used. There were 10 spitzoid melanomas in patients under 20 years of age and 15 in patients > 20 years of age. In both of these age groups the proportion of *BRAF* or *NRAS* mutant cases was the same, with two of 10 mutant cases in patients under 20 years (20%) and three of 15 cases (20%) in patients over 20 years old. When all spitzoid lesions were examined with the cut-off age of 20 years or less, two of 16 (12%) tumours in patients < 20 years of age were mutant, while eight of 39 (21%) cases were mutant in those > 20 years of age. This difference was not significant (P = 0·71).

HRAS mutation and Spitz naevus progression

Next, an assessment of whether Spitz naevi progress to spitzoid melanoma was performed. The *HRAS* mutation frequency in Spitz naevi is approximately 15% based on published data. Assuming that Spitz naevus does progress to spitzoid melanoma and all Spitz naevi have an equal chance of progressing, then 15% of spitzoid melanoma should have an *HRAS* mutation. The frequency of *HRAS* mutation in spitzoid melanoma was therefore assessed, with representative examples shown in

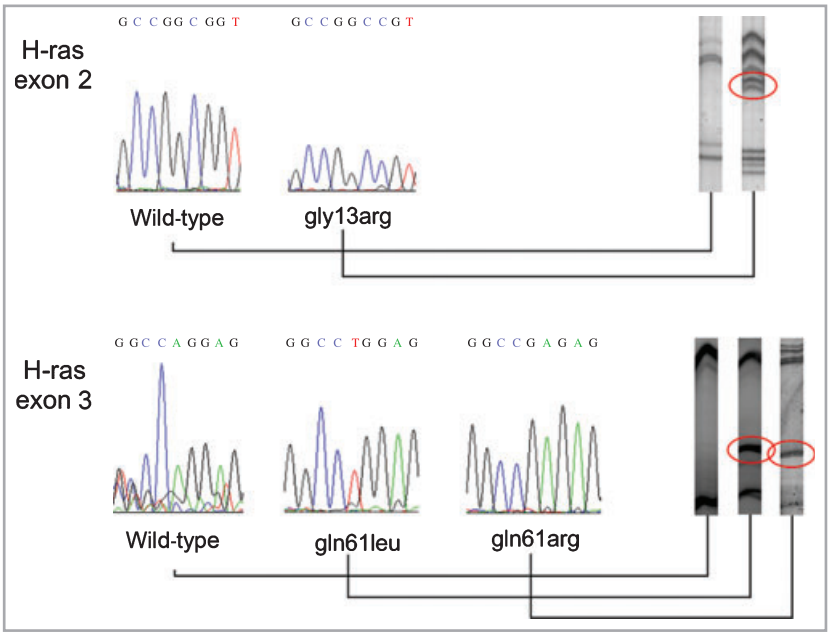

Fig 3. Single-strand conformational polymorphism analysis with accompanying sequencing electropherograms from cases with wild-type and mutant *HRAS* exons 2 and 3. Aberrantly migrating bands are encircled in red.

Figure 3. Of 27 spitzoid melanomas analysed, 21 had complete *HRAS* mutation data. Based on a presumed *HRAS* mutation frequency of 15% in spitzoid melanoma, the binomial probability of finding at least one spitzoid melanoma with an *HRAS* mutation was 0·97. However, no *HRAS* mutations were found, with a probability of 0·03. This probability was so low that an assumption of absent (or at least very rare) *HRAS* mutations in spitzoid melanoma was considered reasonable. In addition, no *HRAS* mutations were found in 22 nonspitzoid melanomas where complete mutation analysis was available. This result supports the notion that Spitz naevus and spitzoid melanoma are distinct categories, which in turn indicates that ambiguous spitzoid tumours cannot represent intermediate degrees of progression from Spitz naevus to spitzoid melanoma.

HRAS mutation and aggressive behaviour in spitzoid tumours

The absence of *HRAS* mutation in spitzoid melanoma suggests that its presence might be a decisive criterion that classifies an atypical spitzoid lesion as Spitz naevus, and thus indicates benign behaviour. Although the numbers are small, there were three *HRAS* mutant cases that could be used to try and falsify this proposal. The mutations were found in one of 12 (8%) Spitz naevi, one of seven (14%) atypical Spitz naevi and one of five (20%) STUMP where *HRAS* mutation analysis was complete. The follow-up information is relatively short, but nevertheless none of these tumours demonstrated malignant behaviour, consistent with classification of an ambiguous spitzoid tumour with *HRAS* mutation as a benign Spitz naevus. The *HRAS* mutant Spitz naevus had an exon 2 mutation and was a compound tumour, 2·4 mm thick, and was considered to show a degree of asymmetry by the reviewing dermatopathologists. This lesion was excised from the temple of a 19-year-old woman, who was disease free after 13 years follow up. The atypical Spitz tumour had an exon 3 mutation and was a dermal lesion that had a thickness of 1·5 mm and showed cytological atypia with a deep mitosis. This lesion was excised from an unknown site in a 36-year-old woman, who was disease free after 8 years follow up. The STUMP had an exon 3 mutation and was a compound lesion, 3 mm thick, and also showed cytological atypia with a deep mitosis. This lesion was excised from the shoulder of a 19-year-old woman, who was disease free after 7 years follow up.

Discussion

This study analysed cases of Spitz naevi, spitzoid melanoma, ambiguous spitzoid tumours and nonspitzoid melanoma and attempted to assess whether these tumours shared a similar molecular basis, whether Spitz naevus progressed to spitzoid melanoma and, lastly, to shed light on the nature of ambiguous spitzoid tumours.

The first question derives from the notion that spitzoid morphology might reflect some underlying molecular similarities. We found that both Spitz naevi and spitzoid melanoma had a low combined frequency of *BRAF* and *NRAS* mutations, and this frequency was not significantly different. However, it was not possible to demonstrate statistical equivalence of mutation frequencies in Spitz naevi and spitzoid melanoma. It therefore remains unclear whether *BRAF* and *NRAS* mutation represents an alteration that will reveal similarities or differences between Spitz naevi and spitzoid melanoma. This dilemma can be resolved only by a much larger study, an aim that is made difficult by the rarity of spitzoid melanoma. Because of these limitations, we decided refocus the analysis by looking at whether spitzoid lesions were different from other more common melanocytic lesions. Both Spitz naevi and spitzoid melanoma showed a combined *BRAF* and *NRAS* mutation frequency that was significantly different from more

common forms of melanocytic tumour, namely common acquired naevus and nonspitzoid melanoma. This is in accord with recent molecular analyses that have revealed a hidden complexity in the molecular pathology of melanoma using a combination of array comparative genomic hybridization with cluster analysis and assessment of *BRAF* and *NRAS* mutations.[7,24] The low mutation frequency in Spitz naevi and spitzoid melanomas is similar to the findings in mucosal melanoma and melanomas from acral and chronically sun-damaged sites.[7,24] These melanomas often harbour c-KIT mutations.[24] It is unknown whether this mutation is present in spitzoid melanoma, this requiring further study. Age has been postulated to account for the difference in *BRAF* and *NRAS* mutation frequencies between spitzoid and nonspitzoid melanomas.[12] No evidence for this was found in the present study, although the number of melanomas in children < 10 years old was small, so it is difficult to make a definitive judgement.

The second key finding, via analysis of *HRAS* mutation, was that Spitz naevi do not appear to be able to progress into spitzoid melanoma. Two previous studies analysed *HRAS* in spitzoid melanoma,[12,16] although Takata *et al.* only had consensus diagnosis of spitzoid melanoma among pathologists in one ambiguous spitzoid case (which had a lung metastasis), for which *HRAS* analysis was not done. Van Dijk *et al.* had complete analysis of *HRAS* mutation hot-spots in 34 cases, with no mutations found. However, the mean age of their cases was 52 years, much older than the Spitz naevus patients in their study (mean age 27 years) and they arguably used more inclusive criteria for spitzoid melanoma – any case composed of spindle and/or epithelioid cells. In our study, cases with such cells were excluded if there was a radial growth phase that clearly placed the melanoma into another category, e.g. superficial spreading malignant melanoma. Nevertheless, taken together, these data are consistent with an absence of *HRAS* mutation in spitzoid melanoma. This leads on to a third important scenario: if Spitz naevi do not progress to spitzoid melanoma then this suggests that all spitzoid tumours are either Spitz naevi or spitzoid melanoma; therefore ambiguous lesions are merely ones that cannot be correctly classified using current diagnostic criteria. However, before casting aside a model in which Spitz naevi progress to spitzoid melanoma, some limitations of the present study should be considered. First, it is possible that there is biased progression of Spitz naevi to spitzoid melanoma, whereby only *HRAS* wild-type cases progress, while *HRAS* mutant cases are somehow protected. Senescence, a characteristic feature of melanocytic naevi,[25] is a critical barrier that prevents progression to melanoma. The molecular basis for this is under intense study. The p16INK4A gene appears to be important and *HRAS* mutation seems to be a powerful inducer of its expression.[26] Also, *in vitro* evidence links *HRAS* mutation to a novel form of senescence related to the 'unfolded protein response'.[27] It is unknown whether this particular type of senescence confers a more effective barrier to malignancy. Assessment of whether *HRAS* mutation provides enhanced protection from progression to spitzoid melanoma cannot be resolved in a correlative study such as this. Another limitation of this study is that the criteria for diagnosing a spitzoid melanoma are not well established. It is therefore quite possible that some lesions that were deemed to be spitzoid melanoma for this study were actually

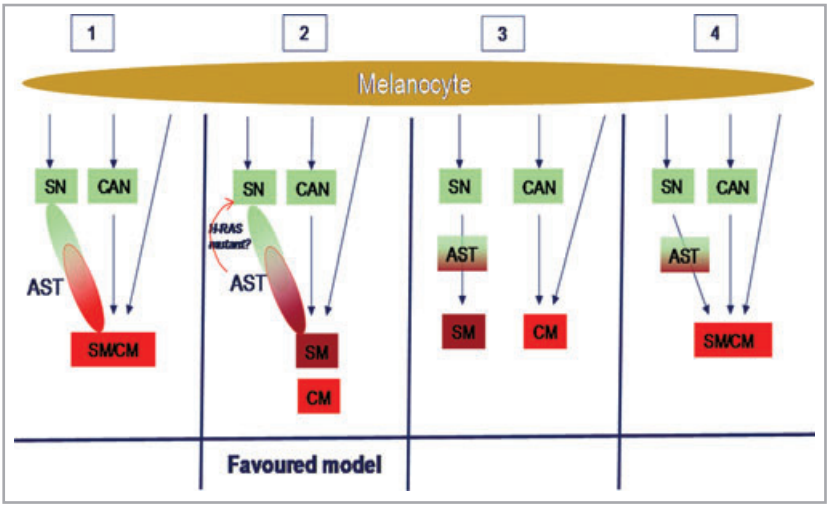

Fig 4. Speculative relationships among spitzoid tumours. A nonexhaustive set of scenarios is presented highlighting possible relationships between common Spitz naevi (SN), spitzoid melanoma (SM), ambiguous spitzoid tumours (AST), common acquired naevi (CAN) and common forms of melanoma (CM). In models 1 and 2, SN do not progress to SM, while in 3 and 4 they do. In models 1 and 2, AST are merely histologically unclassifiable SN or SM, while in model 3 and 4 they are genuine intermediate lesions. These models are further broken down into whether SM and CM are similar (1 and 4) or distinct (2 and 3). Our findings relating to *HRAS* are not consistent with scenario 3 and 4, while the findings related to *BRAF*/*NRAS* are not consistent with scenario 1. The findings therefore suggest scenario 2 to be correct, with *HRAS* mutation being a putative molecular diagnostic marker that classifies AST as SN.

just other forms of melanoma with a prominent population of spindled and epithelioid cells. This would mean that the number of 'true' spitzoid melanomas was less than expected, which in turn would limit the chances of finding an *HRAS* mutant spitzoid melanoma. Analysis of genuine spitzoid melanoma will remain a problem until a more precise definition can be formulated.

In summary, this study demonstrates that a *BRAF*/*NRAS* mutant phenotype is not as important in spitzoid tumours as it is in some other forms of melanocytic tumour. Secondly, this study suggests that Spitz naevus does not progress into spitzoid melanoma, consequently calling into question the position of ambiguous spitzoid tumours as intermediate lesions in a progression pathway. The overall implications are summarized in Figure 4, which highlights a particularly encouraging aspect of this study: if ambiguous spitzoid tumours are likely to be no more than unclassifiable examples of either Spitz naevus or spitzoid melanoma, then molecular markers have the potential to act as important diagnostic signposts for correct classification when histopathological assessment is wanting. For example, our data and other studies[12] would suggest that *HRAS* mutation is a low sensitivity/high specificity marker of Spitz naevus. As the molecular pathology of spitzoid tumours becomes better understood, it is likely that more molecular markers might be identified, leading to further shrinkage of ambiguous spitzoid tumour category. Given that spitzoid lesions account for a disproportionate number of incorrect diagnoses and medicolegal claims,[28] the search for these new molecular markers is urgent.

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Supporting information

Additional supporting information may be found in the online version of this article.

Supplementary Table S1 Complete clinicopathological and mutation analysis data.

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