Doktori (Ph.D.) - értekezés

Újabb prognosztikai faktorok melanoma malignumban

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Tartalomjegyzék

Bevezetés

A melanoma malignum a legrosszabb indulatú bőrdaganat. Előfordulási gyakorisága az európai országokban 10-14/100.000 fő, növekvő tendenciát mutat, 10 évente a gyakoriság megkétszereződik. Korábban elsősorban az 50-60 évesek megbetegedése volt, de napjainkban egyre inkább a fiatalabb korcsoportok irányába tolódik el (1).

Kezelése napjainkban is az időben és megfelelő módon végzett műtét (2). Számos citosztatikus szert alkalmaznak az előrehaladott esetek kezelésére, de ezek egyértelmű hatása nem bizonyított, a kombinált sémákkal is csak 40-49%-os remisszió érhető el (3). Az utóbbi években számos vizsgálat történt az interferonok terápás alkalmazására, de ezek erdményességét bizonyító, nagyszámú beteget érintő prospektiv vizsgálatok még nem fejeződtek be (4).

A betegség lefolyásának megállapítására számos prognosztikai faktort ismerünk és alkalmazunk, de az egyéni betegséglefolyás sok esetben lényegesen eltér a statisztikai adatok alapján várhatótól (5).

Napjainkban az UICC 1987-ben felállított stádiumbeosztása az elfogadott, melyen belül I-IV stádiumot különítünk el, egyes stádiumokban más-más faktorok játszanak prognosztikai szerepet (5, 6, Táblázat).

Mivel az egyéni kimenetel nem mindig egyezik a prognosztikai faktorok alapján várhatóval, célul tűztük ki újabb, esetleg hatékonyabb és objektívebb faktorok keresését és vizsgálatát.

Táblázat: Melanoma malignum prgonózisának megítélésében szerepet játszó jelentősebb tényezők a daganat különböző stádiumaiban (5,6)

Stádium		Stádium meghatározása	Prognosztikai tényező(k)
UICC	Korábbi		
I	I	$pT_{1-2}N_0M_0$	a tumor vastagsága, mélységi, vertikális terjedési szintje, a tumor lokalizációja és a beteg neme
II		$pT_3N_0M_0$	
III		$pT_4N_0M_0$	
	II	$pT_{1-4}N_{1-2}M_0$	a pozitiv nyirokcsomók száma, az elsődleges tumor lokalizációja, valamint annak vastagsága
IV	III	$pT_{1-4}N_{1-2}M_1$	a metasztatikus helyek száma és a metasztázisok lokalizációja

Smith és munkatársai (7) írtak le egy nagyon érzékeny módszert a keringő melanomasejtek, melanociták kimutatására. A módszer a reverse transcriptase - polymerase chain reaction (RT-PCR) segítségével történő, a **tirozináz** enzimet kódoló gén kimutatása. A tirozináz enzim a melanocita sejtekre jellemző, és a malenogenezis két lépcsőjét katalizálja.

Steeg és munkatársai (8) identifikálták az **Nm23**-H1 és -H2 géneket metasztatikus egérmelanoma sejtvonalakban, melyek fontos szerepet játszanak a metasztázisok kialakulásában. Az Nm23-H1 gén szintje mintegy 10X nagyobb volt a nem metasztatizáló sejtvonalakban mint a metasztatizálókban. Számos más rosszindulatú daganat sejtjein is vizsgálták, de nem minden esetben jutottak hasonló megállapításra (9, 10). Florenes és mtsai (11) retrospektiv vizsgálata során forditott arányosságot észleltek a melanoma progressziója valamint az áttétben

mérhető Nm23 szint között.

A bázikus **fibroblaszt növekedési faktor** (FGF) szerepe már régóta ismert és bizonyitott a melanoma patogenezise szempontjából (12,13). Azonban az egyéb fibroblaszt növekedési faktorok szerepe kevésbé tisztázott. Ezért tűztük ki célul a fibroblaszt növekedési faktor 1 (FGF1), valamint receptorának (FGFR1) vizsgálatát és expressziójának esetleges kapcsolatát klinikopatologiaii adatokkal.

Célkitüzések

1. **A tirozináz enzimet kódoló gén, mint a melanocita rendszerre specifikus gén kimutatása.**

1/A Specificitás vizsgálata: A tirozináz gén kimutatása naevusokban, melanomákban ép humán szövetekben, és nem melanocita erdetű malignus daganatokban. Magyarázatot kaphatunk az extrakután kiindulású melanomák eredetére.

1/B. Prognosztikai vizsgálatok: A vérpályába kerülő, hematogén daganat-szóródást eredményező tumorsejtek kimutatása különböző stadiumú melanoma malignumban szenvedő betegek perifériás véréből. A felmerülő kérdések az egyes betegcsoportokban:

-II-III. stádium: Lehetséges-e RT-PCR módszerrel reziduális betegséget kimutatni aktuálisan tumormentes egyénnél?

-III. stádium: A PCR pozitivitás jele lehet-e a korai (egy hónapon belüli) recidivának, valamint a blokk-disszekció során

kerülnek -e tumorsejtek a keringésbe?

-III. stádium: A PCR pozitivás lehet-e rövid időn belüli (6 hónapon) metasztázis jelentkezésének jele?

IV. stádium: A keringő tumorsejtek kimutathatósága összefüggésbe hozható-e a betegség progresszójának mértékével?

2. **Az Nm23-H1 gén vizsgálata melanoma malignum metasztázisaiban.**

A III.-IV. stádiumú melanomás betegek műtéti úton eltávolított metasztázisaiban az Nm23-H1 gén expressziójának meghatározása, majd a beteg nyomonkövetése alapján a kapott eredmények értékelése.

Korreláció keresése az első metasztázis Nm23 szintje és a betegség lefolyása között.

3. **FGF1 gén és FGFR1 gén kimutatása.**

III. és IV. stádiumú melanomás betegekből származó metasztázisokban az FGF1 és FGFR1 gének meghatározása. Az FGF1 és FGFR1 gének előfordulási gyakorisága, expressziója melanomában milyen kapcsolatot mutat a betegség kórlefolyásával?

Anyag és módszerek

A vizsgált szövet- (valamennyi az Institut Paoli-Calmette Pathologiai Osztályának /Marseille/ szövet-archivumából származott) és vérminták (Szent-Margit Kórház, Bőrgyógyászati Osztály, Marseille):

1/A A tirozináz gén meghatározása ép és tumoros szövetekben történt (14, I. sz. melléklet). Műtéti úton eltávolított humán szöveteket dolgoztunk fel. Normál szövetek: nyolc reaktiv nyirokcsomó, melyek közül egyben naevus pigmentosust észleltünk a szövettani vizsgálat során, valamint különböző egyénekből származó bőr, colon, gyomor, máj, lép, tüdő, perifériás ideg, vese, prosztata, here, emlő és ovárium. Tumoros szövetek: két benignus naevus pigmentosus, hét melanoma malignum metasztázist tartalmazó nyirokcsomó, egy liposarcoma, két malignus lymphomás nyirokcsomó, valamint két benignus Schwannoma. A felsorolt tumorok mellett két retinoblastoma sejtvonalat is vizsgáltunk.

Minden szövetminta egyik része rutin szövettani vizsgálatra került, másik felét folyékony nitrogénben lefagyasztottuk és abban is tároltuk a felhasználásig.

1/B Különböző stádiumú malignus melanomás betegektől nyert vérmintákból mutattuk ki a tirozináz gént (15, II. sz melléklet). A betegek a vizsgálatba történő bevonás elött klinikai vizsgálaton, valamint stádium-felmérésen estek át metasztázisaik kiterjedtségének megitélésére. A bevonásuk után rendszeres ellenőrzés alatt álltak, mely laboratoriumi vizsgálatokat és radiologiai ellenőrzést jelentett (a klinikai vizsgálatok a Szent-Margit Kórház Bőrgyógyászati Osztályán /Marseille/ történtek).

A staging vizsgálat alapján a betegeket négy csoportba osztottuk:

- II-III: stádiumú betegek: 10, jelenleg tumormentes melanomás beteg, akiknél a primér tumor vastagsága meghaladta a 2 mm-t. Vérvétel: félévente.
- Stádium III: regionális nyirokcsomó metasztázis.
  18, mütét előtt álló, axilláris vagy inguinális blokkdisszekcióra váró beteg. Vérvétel: a nyirokcsomó eltávolitás napján 8-9 óra között, a műtét előtt, ill. kilenc beteg esetében a mütét alatt, továbbá a mütét utáni napon.
- Stádium III: már korábban regionális nyirokcsomó metasztázissal műtött, jelenleg klinikailag tumormentes 33 beteg. Vérvétel: négyhavonta.
- Stádium IV: távoli metasztázissal rendelkező esetek.
  32 esetben komplett stádium felmérést követően 4 havonként ismételtük a vizsgálatot. 17 esetben a legelső kemoterápiát megelőzően történt a vizsgálat, míg 15 esetben a vérvételt egy órával az esedékes következő kemoterápia előtt végeztük, 12 beteg esetében egy órával a kemoterápia befejezése után is vérmintát vettünk. A progresszió mértékének megitélésére a metasztázisok bidimenzionális méréséből számitott térfogat szolgált.

2. Az Nm23-H1 gén meghatározása melanoma malignum metasztázisaiban (16, III. sz. melléklet) történt. Harminc, (III.st. n:20; IV.st. n:10) metasztatikus melanomás betegből származó műtéti úton eltávolitott, szövettani vizsgálattal igazolt metasztázisokat vizsgáltuk. Ezek lokalizáció szerinti megoszlása: 25 nyirokcsomó,

4 bőr, egy beteg esetében pedig máj. Negativ kontrollként nyolc, egyéb humán szövetet vizsgáltunk (máj, emlő, prosztata, nyirokcsomó, lép és ovarium), valamint 3 benignus naevust.

3. FGF1 és FGFR1 gének vizsgálata humán melanoma szövetben (17, IV. sz. melléklet): 77, műtéttel eltávolitott melanoma malignum metasztatikus szövetmintát vizsgáltunk, melyek közül 59 nyirokcsomó-, 13 kután-, két májmetasztázis valamint 3 primér melanoma volt. Tíz normál szövet ill. pigmentált naevus is feldolgozásra került.

Génexpresszió vizsgálata:

RNS és DNS izolálása (14): A műtéti úton eltávolitott szöveteket a felhasználásig -196°C-on tároltuk. A nukleinsavak izolálására standard guanidinum isothiocyanat/caesium chlorid gradiens ultacentrifugálást alkalmaztunk (18). A 25 ml EDTA-val kezelt perifériás vérből Ficoll izolálással nyert mononukleáris sejtekből is az előbb említett módszerrel állítottuk elő a DNS és RNS-t (15).

Komplementer DNS előállítása(Revers transcriptase RT reakció): A teljes RNS 2 mg-ját használtuk fel a komplementer DNS (cDNS) előállítására Riboclon cDNS szintetizáló rendszerben (Promega Biotec, Franciaország)(14,15).

PCR: A cDNS preparátum 1/8 szolgált alapul a PCR amplifikációhoz. Smtih és mts. által leírt külső és belső primereket alkalmaztunk, két egymást követő, egyenként 30-30 ciklusú amplifikációhoz (14,15). Másik vizsgálatunkban a teljes RNS

felhasználásával RT majd PCR reakciót végeztünk az FGF1 és FGFR1 génre jellemző primérek felhasználásával (17). Az RNS épségét a minden sejten és szöveten expresszálódó GAPDH (glycer-aldehyd-3- dehydrogenase) valamint ß-aktin gén kimutatásával igazoltuk. Mely egyszeri 30 ciklusú amplifikációval történt. A PCR terméket 1,5%-os agarózgél elektoforézis után ethidinum bromiddal festettük meg. A keletkezett reakciótermék ellenőrzésére mindkét esetben Southern blott analizist követően belső szonda alkalmazásával hibridizációt végeztünk, nem radioaktiv kemilumineszcens módszerrel tettük láthatóvá a reakciót (15,17).

Northern blott analizis: Az előzetesen leírt módszerrel izolált teljes RNS 10 mg-ját formaldehid tartalmú denaturált agaróz gélben megfuttatuk, majd Hybond membránba transzferátluk, a gyártó előírásának megfelelően. UV fixaciót követően Nm23-H1 cDNS próbával hibridizációt végeztünk (900bp BamH1 fragment a pMM23-H1 plasmidból), radioizotop (P^{32}) jelöléssel (16). A következő lépésben ugyanazon, dehibridizált membrán GAPDH cDNS hibridizációját követően, denzitometriás leolvasás segítségével az Nm23 mennyiséget a GAPDH mennyiség arányában adtuk meg.

Southern blott analizis: A fagyasztott szövetből izolált DNS-t, EcoR1 restrikciós enzimmel történő emésztése után nylon mebránba transzferáltuk, majd FGFR1 hibridizációs próbához (ECO R1-Bgl-II, pOI10) plasmidot használtunk (17).

***In situ* hibridizáció**: Fagyasztott sorozatmetszeteket használtunk a már előzetesen leírt in situ hibridizációs módszerhez, RT-PCR tesztelt és erős pozitivitás mutató tumormintákból (17,19).

Immunhisztokémia: Fagyasztott metszeteken háromlépcsős ABC technikát alkalmaztunk, az FGFR1 protein lokalizációjának meghatározására. Az első ellenanyag FGFR1 ellenes monoklonalis egér antitest (19B2). A melanoma sejtek párhuzamos jelölésére HMB-45 monoklonális ellenanyagot használtunk (valamennyi IMMUNOTECH, Franciaország) (17).

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Statisztikai módszerek:

1. A tirozináz gén perifériás vérből történő kimutatásakor Fisher féle Exact tesztet, Relativ rizikó, valamint confidencia intervallum indexet és chi ^{2} próbát alkalmaztunk.
2. Az Nm23 -H1 gén vizsgálatokor BMDP package program valamint Kaplan -Meier és Mantel-Cox tesztek szolgáltak statisztikai elemzésünk alapjául.
3. Az FGF1 valamint FGFR1 vizsgálata során kapott eredmények és a klinikopatológia paraméterek közötti összfüggés kimutatására Fisher és Long Rank tesztet használtunk

Megfigyelések

1 Tirozináz gén-expresszió:

1/A A negativ kontrollként használt, egészséges egyének véréből származó mononukleáris sejtekből (esetszám: 6) származó RNS vizsgálatakor egyetlen esetben sem észleltük a tirozináz gén expresszióját. Az ismerten nagyszámú melanocitát tartalmazó szövetekben, 3 normál bőr, 2 benignus naevus valamint 2 melanoma metasztázissal infiltrált nyirokcsomó esetében már az első PCR amplifikáció után erős pozitiv jelet észleltünk. A normál szövetekben a prosztata és

a májszövet kivételével, ahol konzekvensen negativ eredményt kaptunk, a gén expressziója változó volt.

A nem-melanocita eredetű malignus tumoros szövetek vizsgálatakor szintén észleltük a gén expresszióját mely alól kivétel egy Hodgkin kóros nyirokcsomó volt.

1/B A vérminták vizsgálatai során pozitív kontrollként alkalmazott normál bőr, naevus pigmentosus, valamint melanoma metasztázis esetében mindig pozitiv volt a tirozináz gén expressziója.

A 10 egészséges egyénből valamint a négy, nem melanomás disszeminált malignus tumorban szenvedő egyéntől származó vérminta minden esetben negativ eredményt mutatott. A negativ esetek valamint a IV. stadiumú melanomás esetek génexpessziója között észlelt különbség statisztikailag szignifikánsnak bizonyult (0/14-16/32).

-II- III. stádium: A vizsgált 10, csak a primér tumor eltávolításán átesett (tumorvastagság>2mm), nyirokcsomó áttét nélküli beteg közül két esetben észleltük a gén expresszióját. A nyomonkövetés során metasztázis-mentesek maradtak, míg két előzetesen negativ esetben metasztázis kialakulását észleltük.

- III. stádium: A vizsgálat idején regionális nyirokcsomó áttéttel rendelkező esetek (18 beteg) közül 8 esetben találtunk blokk-disszekció előtt génexpressziót. Ezek közül 5 esetben jelentkezett 6 hónapon belül metasztázis, mig a 10, műtét előtt negativ közül csak egynél alakult ki áttét (Fisher féle exact teszt P=0,04). A 9, műtét előtt, alatt és után is vizsgált egyén közül egyetlen esetben sem észleltünk a műtét után pozitivtást azoknál, akik műtét előtt és alatt negativak voltak. A műtét előtti pozitivitást

mutató esetünk a műtét után negativvá vált.

- III. stádium: A 33, korábban blokk-disszekción átesett, jelenleg tumormentes, klinikailag és radiológiailag negativ, de nagy rizikójú csoportba tartozó betegtől 58 vérmintát vizsgáltunk. A pozitiv PCR után 3,8x nagyobb (RR: 3,82; 95% CI: 58-9,22) a 6 hónapon belüli metasztázis kialakulásának valószinűsége, mint negativ teszt után (Ch^2 P=0.002). A primér tumor paramétereinek (vastagság, lokalizáció) figyelembe vételével a relativ rizikó (RR) alig magasabb (RR:5,14; 95%CI:1,04-27,4 és RR:5,15; 95% CI:1,17-22,7).

- IV. stádium: A távoli metasztázissal rendelkező 32 betegtől 93 vérmintát vizsgáltunk. Huszonhárom minta minden szisztémás kezelés nélkül, mig 50 a 4 hetenként esedékes kemoterápia előttről származott. Tizenhat beteg rögtön az első vizsgálatkor pozitiv tesztet eredményezett, az ismételt vizsgálatok során összesen 20 beteg mutatott pozitivitást valamelyik vérmintájában. A pozitiv PCR után a gyors progresszió 4x gyakoribb volt mint a lassú progresszió, vagy a stabil állapot (RR:4,11; 95%CI: 1,93-8,76; Chi^2 P=0,0002). A viszcerális metasztázissal rendelkezőknél gyakoribb volt a pozitiv teszt (41%), mint a csak kután vagy nyirokcsomó áttéttel rendelkezőknél (17%). A metasztázisok helyének figyelembevétele a progresszió szempontjából csak mérsékelten változtatta meg az előzetes eredményeket. (RR:3,99: 95%CI: 1,70-9,39; Chi^2 P =0,0009). A 93 vérmintából 12 azonnal a kemoterápia után készült, 11 esetben negativ eredményt kaptunk.

2 Nm23-H1 gén vizsgálata:

Az Nm23- H1 gén expresszióját a GAPDH mRNS százalékos arányában adtuk meg. A normál szövetek által expresszált Nm23-H1 szint 63%, csaknem megegyezett a normál benignus naevusokban észlelt szinttel. A melanoma csoportban az expresszió lényegesen heterogénebb volt, 7-240% között változott.

- A III. és IV. stádiumú betegek együttes vizsgálata során, ha a betegek által expresszált génszint meghaladta az átlagos értéket (46,9%), jobb prognózissal rendelkeztek mind az annál alacsonyabb értékűek (P=0,08).
- Ha csak a regionális nyirokcsomó metasztázissal rendelkező betegeket (III. stádium) vizsgáltuk és a blokk-disszekciót tekintettük kiindulási pontként, szignifikáns korrelációt észleltünk az első metasztázisban mérhető Nm23-H1 szint és a túlélés között (P=0,035)
-A tünetmentes időszak hossza a túléléssel ellentétben nem mutatott ilyen összfüggést.
- Ha a primér tumor eltávolitását vettük kiindulási alapul (III. IV. st), szintén szignifikáns összefüggést észleltünk az első metasztázis megjelenési ideje és a benne mérhető Nm23-H1 szint között.
- Ha csak a primér tumorral rendelkező eseteket (I. st) vettük figyelembe, a vizsgált első metasztázisban talált Nm23 expresszió a betegség progressziójára is utalt, a középértéket meghaladóknál lassúbb lefolyást észleltünk (P=0,004.).
- A viszcerális metasztázissal rendelkezők átlagos Nm23 szintje alacsonyabb volt (31%) mint a csak izolált nyirokcsomó metasztázissal rendelkezőké (51%), de az eltérés nem volt szignifikáns.

3 Fibroblaszt növekedési faktor meghatározása:

- A RT-PCR módszerrel FGF1 gén és FGFR1 gén kimutatható a melenomák többségében (69/77, 90%, valamint 68/77, 88%). Nyolc tumor esetében észleltünk eltérést az FGF1 és FGFR1 expressziója között. Négy esetben mindkettő negativ volt. A normál bőr és a benignus naevusok konzekvensen expresszálták mindkét gént.
- Immunhisztokémiai vizsgálattal az FGFR1 protein erős pozitivitást adott a reaktiv sztromasejtekben, míg a tumorsejtekre gyenge festődés volt jellemző.
- *In situ* hibridizációval az FGFR1 RNS termelése mind a reaktiv, mind a tumoros sejtekben észlelhető, de utóbbiakban alacsonyabb mértékben.
- A Southern-blott analízis során a melanomás tumorok Eco RI emésztést követően 6 kb és 2,5 kb csíkot eredményeztek, hasonlóan a normál bőrhöz és a benignus naevusokhoz. A genomkárosodásra utaló hiányt vagy többlet elváltozást nem észleltünk.
- Fisher teszt alkalmazásával nem találtunk a primér tumor vastagsága, valamint a RT-PCR módszerrel meghatározott szint között összfüggést (P=0,7 FGF1; P=0,47 FGFR1). Long Rank teszt sem mutatott korrelációt a metasztázis eltávolitás utáni túléléssel (P=0,29 FGF1; P=0,8 FGFR1), az ismételt relapszussal (P=0,66 FGF1; P=0,99 FGFR1), valamint a primér tumor és az első metasztázis megjelenésének idejével (P=0,09 FGF1; P=0,58 FGFR1).

Megbeszélés, új megfigyelések

1/A Tirozináz gént kimutatható számos szervben, igy bőrben nyirokcsomóban antrumban, colonban, vesében, tüdőben, here, ovarium, emlő és perifériás ideg szövetében. Számos malignus és benignus folyamatban, naevusok, emlőcarcinoma, malignus lymphoma és schwannoma szöveteiben is megtalálható. Ezen módszerünkkel meghatározott sejtek megfelelhetnek jól differenciált melanocitáknak, melanocita prekurzoroknak, vagy Schwann sejteknek, melyek melanocita differenciáló potenciállal rendelkeznek. A primér extrakután melanomák eredetének ezen lehetőségek egyike szolgálhat magyarázatul (14). Tudomásunk szerint ez az első olyan tanulmány mely a tirozináz gén kimutatását végzi különböző humán szövetekben.

1/B Melanoma malignumban szenvedő betegek bármelyik stádiumában kimutatható keringő melanomasejt, RT-PCR módszerrel. Az egészséges egyének, vagy egyéb nem melanocitás áttétes tumoros betegeknél nem észlelhetők.
Megállapitható, hogy a kimutatható keringő melanociták jelenléte gyors progresszióra utal a III . stádiumú nyirokcsomó metasztatikus esetekben, rövid időn belüli relapszust jelent a magas rizikójú, bár tünetmentes esetekben, és gyors, súlyos progresszió jele lehet a távoli metasztázissal rendelkező esetekben. Ezen vizsgálat alapján felmerül az alább felsorolt csoportba tartozó betegek szisztémás kezelésének szükségessége (15). Ez az első olyan prospektív vizsgálat, mely a tirozináz gén PCR módszerrel történő kimutatását, mint a melanoma malignumos

beteg prognosztikai szempontból meghatározó faktorát értékeli.

2 A malignus folyamatokban észlelt heterogén eloszlású Nm23 átlagos szint magasabb volt mint a normál szövetekben. A metasztatikus szövetben magasabb Nm23 szintet mutató esetekben hosszabb volt a mütét utáni túlélés, izolált nyirokcsomó érintettség esetén pedig szignifikáns volt a pozitiv korreláció. A primér tumor eltávolitása, valamint az első nyirokcsomó metasztázis megjelenési ideje és ezen első metasztázisban mérhető Nm23 szint között is pozitiv korreláció volt. Jelen vizsgálat mutatja először hogy az Nm23 szint prognosztikai tényezőként használható metasztatikus melanomában (16).

3 Megállapitottuk, hogy FGF1 és FGFR1 gyakran koexpresszálódik malignus melanomában, mely aberrált autokrin és parakrin mechanizmusnak felelhet meg. Nem találtunk korrelációt a klinikopatologiai paraméterekkel igy ezek alapján ezen gének kimutatása nem alkalmazható prognosztikai paraméterként melanomában (17).

Köszönetnyílvánítás

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Összefoglalás

Jelen munkámban újabb prognosztikai faktorok lehetöségét vizsgáltam, melyek segítségével a melanoma malignumban szenvedő betegek betegséglefolyása könnyebben és biztonságosabban meghatározható, ilymódon a szisztémás kezelés indikációja hamarabb felállítható.

Új megfigyelések:

1. Tirozináz gén, eddig a melanocita rendszerre specifikusnak tartott gén számos egyéb szövetben is megtalálható.
2. A malignus melanomás betegek szérumából kimutatható tirozináz gén elsősorban a III. és IV stádiumú betegeknél gyorsabb progressziót jelent, prognosztikai faktorként használható.
3. Az Nm23-H1 gén szintje nemcsak jele lehet a gyors progressziónak, hanem használható, mint prognosztikai faktor disszeminált melanomás esetekben.
4. FGF1 és FGFR1 gén gyakran expresszálódik melanomában, mely aberrált autokrin és parakrin mechanizmus eredménye. A génexpresszió klinikopatologiai korrelációt nem mutat.

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Tyrosinase Gene Expression in Human Tissues

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The occasional occurrence of primary extra-cutaneous malignant melanomas (MM) has led to the hypothesis that melanocytes derived from the neural crest may be arrested in their migration and may undergo an in situ malignant transformation. However, aggregates of nevus cells have only rarely been identified by histological examination in a few organs other than skin and eye. Tyrosinase is a melanin biosynthetic enzyme that is considered one of the most specific markers of melanocytic differentiation. We have attempted to detect cells committed to the melanocytic lineage, in human tissues, by means of tyrosinase gene expression. Total RNA was extracted from normal and neoplastic tissues and analyzed using a highly sensitive reverse transcription PCR assay with primers specific for the tyrosinase gene. Peripheral blood mononuclear cells (PBMC) from healthy subjects were used as negative controls. Tyrosinase transcripts were identified in a wide range of normal organs such as skin, lymph nodes, antrum, colon, kidney, lung, testis, ovary, breast, and peripheral nerve. Tyrosinase RNA was also detected in neoplastic samples including benign cutaneous nevi, lymph nodes involved by MM, breast carcinoma, liposarcoma, malignant lymphoma, and schwannoma. PBMC from patients with metastatic MM were also positive, while no positivity was detected in blood specimens from patients with other cancers.

Therefore, it appears likely that cells expressing the tyrosinase gene are present in a wide range of human tissues. Although these cells still have to be accurately identified, one could propose that they might correspond to either fully differentiated melanocytes, melanocytic precursors, or Schwann cells bearing potentialities of melanocytic differentiation. Occurrence of at least some cases of primary extra-cutaneous MM may be ascribed to any one of these possibilities.

Key words: Melanoma, Tyrosinase, Polymerase chain reaction

INTRODUCTION

Although melanocytes are derived from the neural crest and usually located in the skin, the eye, and the nasopharyngeal tract, aggregates of nevus cells forming blue nevus-like lesions have been occasionally reported in lymph nodes (Erlandson and Rosen, 1982; Vittal Shenoy et al., 1987) and much more rarely in a few other organs such as prostate and cervix vagina (Goldmann and Friedman, 1967; Jao et al., 1971).

Approximately 4% of the patients with malignant melanoma (MM) present with a primary lymph node involvement (Das Gupta et al., 1963). Among these cases, spontaneous regression of a cutaneous MM can sometimes be demonstrated. However, in a few remaining cases where no history of cutaneous tumor is documented, it is tempting to speculate that ectopic benign melanocytes such as those observed in lymph nodes may offer an alternate noncutaneous anatomic source for MM.

The aim of this study was to investigate the presence of melanocytic cells throughout human tissues in order to check the hypothesis that at least some unusual locations of primary extra-cutaneous MM could be related to an in situ transformation of neural crest cells arrested in their migration. Since most human tissues are histologically devoid of melanocytic component, we have attempted to detect melanocytic cells by the means of a highly sensitive and specific reverse transcriptase-polymerase chain reaction (RT-PCR) assay. Several melanin biosynthetic enzymes, acting as tissue-specific proteins, can be used as markers of melanocytic differentiation. Tyrosinase is one

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of these enzymes. The organization of the tyrosinase gene allowed us to perform a RT-PCR assay using primers located in different exons (Kwon et al., 1987; Smith et al., 1991). Tyrosinase gene expression was detected in a wide range of normal and neoplastic human tissues.

METHODS

Tissue Sampling

Biopsy samples from human tissues were surgically removed. Neoplastic specimens included two benign nevi, two lymph nodes involved by MM, one sample of soft tissue infiltrated by liposarcoma, two samples of mammary gland with ductal carcinoma, two lymph nodes involved by malignant lymphomas, and two benign schwannomas. In addition to biopsies, two neuroblastoma cell lines were also analyzed.

Nonneoplastic tissues consisted of eight lymph nodes displaying minor inflammatory lesions such as sinusal histiocytosis. Only one out of those eight samples showed evidence of benign nevus cells in the capsule (Fig. 1). Histologically normal specimens from each of the following organs removed from different patients were also included: skin, colon, stomach, liver, spleen, lung, peripheral nerve, kidney, prostate, testis, breast, and ovary. A part of each sample was processed for conventional histological analysis and the other part was frozen and stored at −70°C.

In addition, peripheral blood mononuclear cells (PBMC) were isolated from six healthy donors, from 12 patients presenting with metastatic MM, and from nine patients with nonmelanocytic cancers of advanced stage.

cDNA Synthesis

Total RNA was extracted by a standard guanidium isothiocyanate/caesium chlorid method. For the synthesis of the first strand of cDNA, 2 μg of total RNA were used with the Riboclone cDNA synthesis system (Promega Biotec, Madison, WI) using the reaction conditions specified by the supplier. Briefly, RNA was first incubated for 5 min at 70°C with 0.8 μg/μl of oligo(dT) primer and then at 42°C for 1 hr with 25 μl of a reaction mixture containing 1 mM of each dNTP, 10 mM DTT, 25 U RNAs in ribonuclease inhibitor, 15 U avian myeloblastosis virus reverse transcriptase, 4 mM sodium pyrophosphate, and 0.5 mM spermidin.

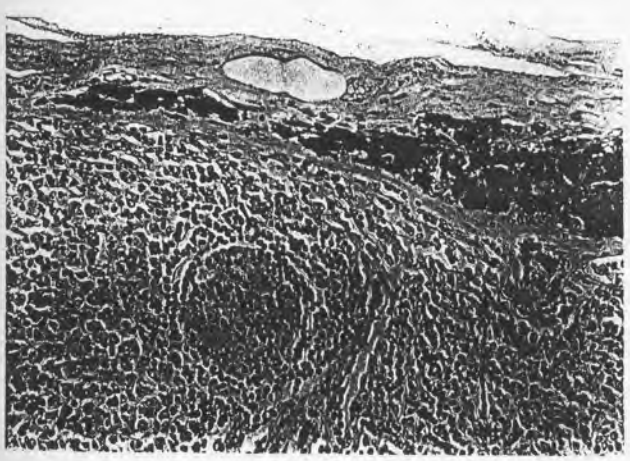

Fig. 1. Histological identification of benign melanocytes by PS100 immunostaining in the capsule of one tyrosinase positive lymph node (PCR analysis shown in Fig. 3).

PCR Analysis

PCR amplification was performed with one-eightieth of the cDNA preparation. The sequence and the location of outer and nested tyrosinase primers was previously described (Smith et al., 1991). cDNA was mixed with 100 μl of a PCR mixture containing reaction buffer and Taq polymerase supplied by Perkin Elmer Cetus (Norwalk, CT). The PCR cycle profile was denaturation at 94°C for 1 min (2 min for the first cycle), annealing at 55°C for 2 min, and extension at 72°C for 2 min (10 min for the last cycle). Thirty PCR cycles were carried out with the outer primers and then 5 μl of PCR products were reamplified for a further round of 30 cycles with nested primers. In order to avoid contamination, pre- and post-amplification steps were performed in separate rooms, and components of the PCR were manipulated under a laminar hood with positive displacement pipettes.

PCR products were visualized in ethidium-bromide stained 1.5% agarose gels. The integrity of each RNA sample was checked by reverse transcription followed by PCR with primers for the human GAPDH gene (sense: 5′- AAC GGA TTT GGT CGT ATT GGGC -3′ and antisense: 5′- AGG GAT GAT GTTCTG GAG AGCC -3′), which is expressed in almost all types of cells and tissues.

Southern Blotting

After electrophoresis, PCR products were transferred onto Hybond nylon membranes (Amersham, UK) as indicated by the manufacturer. The filters were prehybridized for 2 hr at 45°C in 5 × SSC containing 0.1% laurylsarcosin, 0.02% SDS, and 0.5% blocking reagent (Boehringer, Mannheim, Germany). Hybridization was carried out overnight at 45°C in the same solution, using an oligonucleotidic tyrosinase probe 5′- GAG GGA CCT TTA CGG CGT AAT-3′ located between the internal primers (Kwon et al., 1987; Smith et al., 1991). The probe was nonradioactively labeled with DIG-dUTP using a 3′ tailing kit (Boehringer) according to the supplier's recommendations. After hybridization, the filters were washed twice for 10 min at room temperature in 2 × SSC, 0.1% SDS, and then twice for 10 min at 55°C in 0.1 × SSC, 0.1% SDS. After washing, the hybridized blots underwent a revelation processing using a chemiluminescence detection kit (Boehringer) and were then placed under X-ray film for 10 to 20 min to develop.

A positive hybridization signal was always obtained when a band of the expected size was visualized by ethidium bromide on agarose gels, thereby ensuring the specificity of the PCR products.

RESULTS

Controls

mRNA samples extracted from PBMC were used as negative controls, since we would not expect melanocytes to be present normally in the peripheral blood of healthy sub-

Fig. 2. Ethidium bromide gel showing the negativity of PBMC cDNAs from healthy subjects after two rounds of amplification with outer and nested primers of the tyrosinase gene (Lanes C to G). The same cDNA samples were successfully amplified with primers specific to the GAPDH gene (Lanes H to M). W: molecular weight marker; A: negative control without cDNA; B and H: positive control (MM) amplified with both tyrosinase (B) and GAPDH (H) primers.

jects. All six PBL samples were indeed found to be negative (Fig. 2). Each negative sample was analyzed at least twice.

As expected, every tissue supposed to contain high amounts of melanocytes, i.e., three samples of normal skin, two benign nevi, and two lymph nodes involved by MM, gave strong positive signals even after the first 30 cycles of amplification. Dilution experiments were then performed by diluting cDNA from MM samples used as positive controls in DNA from PBL samples. The sensitivity that we obtained was about $1/10^4$ with outer primers alone and about $1/10^6$ with nested primers.

Normal and Neoplastic Human Tissues

Results are summarized in Tables 1 and 2, and illustrated in Figure 3. Five out of eight lymph nodes analyzed were positive. Only one out of the five positive nodes contained benign nevus cell aggregates upon histological examination (Fig. 1). Normal skin, as expected, was positive for tyrosinase gene expression. Results regarding other normal tissues were surprising, showing a wide range of positivity in various organs such as testis, antrum, colon, spleen, ovary, lung, kidney, breast, and peripheral nerve (Table 1).

However, it is noteworthy that different samples of the same organ obtained from distinct patients were not all positive, i.e., positivity depended not only on the organ but also on the patient.

Prostate and liver samples remained negative. Among the neoplastic samples, a clear positivity was seen in two cases of breast carcinoma, one liposarcoma, one lymph node involved by malignant lymphoma, and two schwannomas of the acoustic nerve. One out of two neuroblastoma cell lines was also positive (Table 2).

Eight out of 12 PBMC samples from patients with metastatic MM were positive, while no positivity was detected in nine blood samples from patients presenting with other types of cancer (Table 3).

TABLE 1. Tyrosinase Gene Expression in Nonneoplastic Tissues

Site[a]	Tyrosinase RNA[b]
Skin	3/3 (++)
Lymph node	5/8 (+)
Antrum	1/2 (+)
Colon	1/2 (+)
Liver	0/2
Kidney	1/1 (+)
Lung	1/1 (+)
Testis	1/1 (+)
Prostate	0/1
Ovary	1/1 (+)
Breast	1/1 (+)
Peripheral nerve	1/2 (+)
Spleen	1/2 (+)

[a]For each organ, the analyzed samples were removed from different patients.
[b]Results of tyrosinase RNA detection are scored as fractions indicating the number of positive samples compared with the total number of analyzed samples. (++): strong positivity (signal detected after the first round of amplification: 30 cycles); (+): positivity detected after the second round of amplification only (30 + 30 cycles). Positive GAPDH RNA detection was obtained prior to tyrosinase RNA detection for all the included samples.

DISCUSSION

Tyrosinase catalyzes the conversion of tyrosine to Dopa and of Dopa to dopaquinone, leading to the formation of melanocytic pigments. Therefore, expression of the tyro-

TABLE 2. Tyrosinase Gene Expression in Neoplastic Samples

Site[a]	Histological Diagnosis	Tyrosinase mRNA[b]
Skin	Benign nevus	2/2 (++)
Lymph node	Malignant melanoma	2/2 (++)
Lymph node	Malignant lymphoma	1/1 (+)
Lymph node	Malignant lymphoma (Hodgkin's)	0/1
Breast	Ductal infiltrating carcinoma	2/2 (+)
Soft tissue	Liposarcoma	1/1 (+)
Acoustic nerve	Schwannoma	2/2 (+)
SK-N-MC cell line	Neuroblastoma	(+)
SK-N-SH cell line	Neuroblastoma	(−)

[a]For each organ, the analyzed samples were removed from different patients.
[b]Results of tyrosinase RNA detection are scored as fractions indicating the number of positive samples compared with the total number of analyzed samples. (++): strong positivity (signal detected after the first round of amplification: 30 cycles); (+): positivity detected after the second round of amplification only (30 + 30 cycles). Positive GAPDH RNA detection was obtained prior to tyrosinase RNA detection for all the included samples.

in some but not all biopsy samples from the same organ. In this context, prostate and liver may not prove to be negative if a larger number of samples were examined.

In the second hypothesis, the cells expressing the tyrosinase gene throughout the human body could be melanoblastic precursors bearing potentialities of melanocytic differentiation. It has been demonstrated in some species that neural crest cells differentiate into melanocytes only when they have reached the epidermis, while neural crest cells arrested in the dermis do not achieve their maturation and represent a potential reservoir of melanoblasts (Teillet, 1971).

Moreover, in situ hybridization experiments have shown that isolated melanoblasts expressing the tyrosinase gene can be identified before they produce detectable amounts of pigment (Beermann et al., 1992). Cell-type specificity and developmental regulation of the expression of the tyrosinase gene in melanocytes are indeed achieved by upstream regulatory sequences (Muller et al., 1988; Ruppert et al., 1988). It is therefore possible that tyrosinase transcripts detected in our study are produced by "melanoblastic" cells that synthesize an inactive tyrosinase protein and are, therefore, devoid of functional tyrosinase activity.

The last hypothesis may be that tyrosinase mRNA is not produced by cells belonging to the melanocytic lineage but rather by peripheral neurons or Schwann cells, which represent a common component of almost all human organs. This hypothesis is indeed supported by the observation of developmental potentialities among cultures of neural crest cells showing the existence of a pluripotent precursor of Schwann cells, satellite cells, neurons, and melanocytes (Dupin et al., 1990). It has also been shown in hamsters that inactive tyrosinase protein can be detected in some neural crest-derived cells, such as satellite cells of spinal ganglia and Schwann cells (Haninec and Vachtenheim, 1988), which normally do not produce melanosomes. The fact that peripheral nerve and Schwannoma were positive in our study tends to favor this hypothesis. However, since most of the normal counterparts of neoplastic samples in our study were positive, the interpretation of tyrosinase RNA expression in neoplastic tissues requires caution.

The unexpected positivity of some neoplastic samples, such as liposarcoma, raises the question whether the PCR method has targeted the true tyrosinase RNA and not another transcript with a closely related nucleotide sequence. As far as we are aware, tyrosinase-related peptides (TRPs) are the only molecules sharing a significant homology with tyrosinase (Halaban and Moellmann, 1990; Jackson et al., 1992; Shibata et al., 1992), but our primers were absent in their cDNA sequences.

Moreover, since the expression of these two loci is also pigment-cell specific (Shibata et al., 1992), a hypothetic cross-reaction with these cDNA segments would not explain unexpected positivities among neoplastic samples histologically devoid of melanocytic component. The hypothesis that such false positivities might have occurred seems unlikely for at least two reasons: (1) the specificity of the PCR is considerably improved by the use of nested primers and (2) the specificity of the amplified products was ensured by their expected size and by Southern blot hybridization with a tyrosinase probe under high stringency conditions. Thus, it appears that detection of tyrosinase cDNA in nonmelanocytic neoplastic samples cannot be explained by a lack of specificity in our experiments.

In a previous report, Smith et al. (1991) reported that some false positive results were observed when 40 rather than 30 cycles were performed in the second round of PCR. According to these authors, false positivities may be due to very low levels of contamination or to a phenomenon of "illegitimate transcription" (Chelly et al., 1989). Although in the present study we never used more than 30 cycles in each round of PCR, and we never observed obviously "false" signals, such as positivity in PBMC of healthy patients, the possibility that "illegitimate transcription" could be a potential source of the tyrosinase transcript cannot be completely ruled out.

There is yet another way to explain unexpected positivities. It is possible, for instance, that positive tumors may contain small amounts of residual normal tissue, which would be responsible for tyrosinase expression in a manner similar to the normal counterparts of neoplastic tissues. Especially for lymph nodes, one can presume that the capsule may not be completely involved and may still contain remnants of ectopic melanocytes.

To further investigate this point, RT-PCR was performed on PBMC from patients presenting with metastatic MM and from patients bearing other types of advanced stage cancers. We have assumed that at least some of these blood samples were likely to contain circulating malignant cells. Our results (Table 3) corroborate those of Smith et al. (1991) and show that positive signals are present only in PBMC from patients with MM, and not in any of the blood samples from patients with other cancers. This is in accordance with the hypothesis that tyrosinase trancripts cannot be produced by nonmelanocytic circulating malignant cells, which are isolated from their environment of residual or reactive tissue.

Finally, despite the limitations of the PCR experiments, the present study may be an opening toward the understanding of at least some cases of MM developing primarily in extra-cutaneous locations such as lymph nodes (Das Gupta et al., 1963), lung (Bagwell et al., 1989), ovary (Ueda et al., 1991), and other uncommon sites, even if one must keep in mind that the majority of these cases probably represents metastases of unknown origin. The cells detected in this study are indeed capable of expressing the tyrosinase gene and are therefore committed at least to very early steps of the melanocytic differentiation pathway. Disregarding the question of the accurate nature of these cells, which needs to be further elucidated, one can presume that they might represent a potential source of MM.

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II. sz. melléklet: **Battyáni Z,. Grob JJ, Xerri L, Noe Ch, Zarour H, Houvaeneghe G et al.: Polymerase chain reaction detection of circulating melanocytes as a prognostic marker in patients with melanoma. Arch. Dermatol.1995;131:443-447.**

STUDY

Polymerase Chain Reaction Detection of Circulating Melanocytes as a Prognostic Marker in Patients With Melanoma

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Background and Design: Polymerase chain reaction (PCR) detection of circulating tumor cells from malignant melanoma (MM) was recently described, but the prognostic value of this method in the treatment of patients with MM remained unclear. In the present prospective study, blood samples (n=193) were collected from 93 patients with MM: 10 stage I patients after primary tumor resection, 18 patients with regional lymph node metastases before node resection, 33 disease-free but high-risk patients (previously treated for node metastases), and 32 patients with distant metastases. Circulating melanocytes were detected using a reverse transcriptase PCR method that analyzes tyrosinase gene expression. All patients were kept under regular surveillance.

Results: The PCR assay was always negative in normal individuals and in subjects with non-MM metastatic cancer, while it was positive in 16 of 32 patients with disseminated MM. Five of eight patients who were PCR-positive before node dissection vs one of 10 who were PCR-negative relapsed within 6 months after surgery. In high-risk but apparently disease-free patients, the risk of relapse within the next 6 months was 3.8 times higher after a positive test result. In patients with distant metastases, a positive PCR predicted rapid disease progression.

Conclusions: These data suggest that PCR detection of circulating melanocytes can be considered as a marker for rapid postoperative relapse after node dissection in patients with MM with regional node metastases, for short-term relapse in high-risk disease-free patients, and for rapid and severe progression in patients with distant metastases. This test may have a crucial interest in the treatment of patients with MM.

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IN PRIMARY malignant melanoma (MM) (stage I), the best prognostic factor is tumor thickness.[1] In patients with lymph node metastasis (stage II), prognosis is correlated with the number of positive lymph nodes, site of primary MM, and tumor thickness.[1] In patients with distant metastatic sites (stage III), the best predictive factors are number of metastatic sites and location of metastases.[2] However, all these factors are of limited value and the behavior of a given MM in a given patient remains somewhat unpredictable. To improve the treatment of patients with MM, new tools for staging and surveillance are needed.

A highly sensitive method for the detection of circulating melanocytes in patients with melanoma, has recently been described[3] and confirmed.[4] This polymerase chain reaction (PCR) method is based on complementary DNA (cDNA) amplification of the tyrosinase gene,[5] which is considered as an enzyme specific for melanocytic differentiation.

The purpose of the present prospective study was to assess the prognostic value of the presence of circulating melanocytes in patients with melanoma. The PCR results in 93 patients were thus correlated with follow-up data.

RESULTS

CONTROLS

As expected, the PCR test was positive in all three normal skin samples as well as in three benign melanocytic nevi and four MMs. These latter specimens gave a strongly positive band after the first round (30 cycles) of amplification using external primers only. Neither the 10 periph-

See Materials and Methods on next page

MATERIALS AND METHODS

RNA AND cDNA PREPARATION

Blood samples and biopsy specimens were prepared as follows: 15 to 25 mL of peripheral blood was placed on a Lymphoprep (J-BIO, Les Ullis, France) layer and centrifuged at 800*g* for 5 minutes at room temperature. Peripheral blood mononuclear cells (PBMC) were collected at the interface resuspended in a washing buffer (TRIS buffer [10 mmol/L at a pH of 7.6], magnesium chloride [5 mmol/L], and sodium chloride [10 mmol/L]); and then immediately used for RNA extraction. Half of each fresh tissue biopsy specimen was analyzed by conventional histologic processing and half was immediately frozen in liquid nitrogen. The PBMC or ground tumor tissue were dissolved in guanidinum thiocyanate, left for 15 minutes at 50°C, and then centrifuged for 5 minutes at 3000 rpm. The solution was placed on a layer of cesium chloride and centrifuged for 20 hours at 20°C. The RNA pellet was aspirated from the bottom of the tube and dissolved in diethylpyrocarbonate (DEPC) (Sigma Chemical Co, St Louis, Mo) water.

Two milligrams of RNA were used for the synthesis of the first strand of cDNA with the Riboclone cDNA system (Promega Biotec, Madison, Wis). Briefly, RNA was first incubated for 5 minutes at 70°C with 0.8-mg/mL oligo (dT) primer in a total volume of 5 μL and then for 1 hour at 42°C with a reaction mixture containing 1-mmol/L each of deoxynucleotide triphosphate (dNTP), 10-mmol/L dithiotreitol (DTT), 25 U of RNAs in ribonuclease inhibitor, 15 U of avian myeloblastosis virus reverse transcriptase, 4-mmol/L sodium pyrophosphate, and 0.5-mmol/L of spermidin in a total volume of 25 μL.

PCR PROCEDURE

The PCR amplification of one eightieth of the cDNA preparation was done using the outer and nested tyrosinase primers described by Smith et al[3]:

HTYR1 = TTGGCAGATTGTCTGTAGCC (outer sense)
HTYR2 = AGGCATTGTGCATG CTGCTT (outer antisense)
HTYR3 = GTCTTTATGCAATGGAACGC (nested sense)
HTYR4 = GCTATCCCAGTAAGTGGACT (nested antisense)

The cDNA was mixed with 100 mL of a PCR mixture containing reaction buffer and *Taq* polymerase (Perkin-Elmer-Cetus, Norwalk, Conn). Each PCR cycle included denaturation at 94°C for 1 minute (2 minutes for the first cycle), annealing at 55°C for 2 minutes, and extension at 72°C for 2 minutes (10 minutes for the last cycle). Thirty PCR cycles were carried out with the outer primers and then 5 mL of PCR products were reamplified for a further round of 30 cycles with nested primers. To avoid contamination, the preamplification and postamplification steps were performed in separate rooms, and components of the PCR were handled under a laminar-flow hood with positive displacement pipettes. The PCR products were visualized in ethidium bromide–stained 1.5% agarose gels.

SOUTHERN BLOTTING

After electrophoresis, PCR products were transferred onto nylon membranes (Hybond, Amersham, England) as indicated by the manufacturer. The filters were prehybridized for 2 hours at 45°C in a medium containing 0.1% laurylsarcosin, 5× standard saline citrate, 0.02% sodium dodecyl sulfate, and 0.5% blocking reagent (Boehringer, Manheim, Germany). Hybridization was carried out overnight at 45°C in the same medium, using an internal oligonucleotidic tyrosinase probe 5′-GAG GGA CCT TTA CGG CGT AAT-3′. The probe was nonradioactively labeled with digoxigenin-deoxyuridine triphosphate (DIG-dUTP), using a 3′-tailing kit (Boehringer), according to the supplier's recommendations. After hybridization, the filters were washed twice for 10 minutes at room temperature in 2× standard saline citrate, 0.1% sodium dodecyl sulfate, and then twice for 10 minutes at 55°C in 0.1× standard saline citrate, 0.1% sodium dodecyl sulfate. After washing, the hybridized blots underwent a revelation processing using a chemiluminescence detection kit (Boehringer) and were then placed under X-ray film for 10 to 20 minutes to develop. A positive hybridization signal was always obtained when a band of expected size was visualized by ethidium bromide on agarose gels, thereby ensuring the specificity of the PCR products.

CONTROLS

Integrity of each mRNA sample was checked by reverse transcription followed by PCR with primers for the human GAPDH gene (sense: 5′-AAC GGA TTT GGT CGT ATT GGGC-3′ and antisense: 5′-AGG GAT GAT GTT CTG GAG AGCC-3′), which is expressed in almost all cells and tissues.

eral blood samples from normal individuals nor the four non-MM metastatic cancers gave positive PCR signals, even after two rounds (30 plus 30 cycles) of amplification using both external and nested primers. Sensitivity obtained by dilution experiments was 10^{-4} with external primers only and 10^{-6} after two rounds of complete nested amplification.

The PCR results in negative controls were significantly different from those observed in patients with stage III disease with MM: zero of 14 subjects without MM (10 normal patients plus four subjects with other cancers) vs 16 of 32 in the first blood sample obtained in patients with metastatic MM.

PATIENTS WITH STAGE II DISEASE BEFORE LYMPH NODE DISSECTION

Of the eight patients who were PCR positive immediately before lymph node dissection, five relapsed within 6 months: three in the same site, one in distant lymph nodes, and one in a visceral site. Conversely, only one of the 10 patients who were PCR negative relapsed in a distant visceral site (five of eight vs one of 10, Fisher's Exact Test: $P=.04$). The mean duration of follow-up was 16 months (range, 6 to 24 months).

In the nine patients (patients 1 through 9 [**Figure 1**]) who were also tested for PCR during and

The PCR assay was validated using negative and positive controls. The negative controls were blood samples from 10 normal individuals and four non-MM metastatic tumors (squamous cell carcinoma of the vulvae, liposarcoma, breast adenocarcinoma, and malignant lymphoma). The positive controls were biopsy samples from normal skin, melanocytic nevi, and metastases of MM as well as blood samples from patients with disseminated MM. Each panel of analyzed patients included at least one negative (PBMC of healthy subjects) and one positive control (MM tumor). The PCR analysis was repeated at least twice on each sample.

The sensitivity of the PCR experiments was evaluated by diluting RNA from MM tumor samples in the PBMC RNA from healthy subjects.

STUDY DESIGN

In the week before inclusion in this prospective study, patients underwent clinical examination and computed tomographic scan of the pelvis, abdomen, chest, and brain. After inclusion, patients were kept under regular surveillance involving repetitive clinical examination (every 2 months) and radiological imaging (third month, sixth month, and at a 4-month interval). Based on initial findings, patients were divided into four groups designed to assess four different applications of PCR testing.

Patients With Stage II Before Lymph Node Dissection

The aim was to determine if PCR before lymph node dissection is a marker of risk for short-term recurrence within the first months after surgery. Eighteen patients with MM with regional lymph node enlargement (stage II) who were scheduled for lymphadenectomy in the axillary or inguinal area, but who were free of other metastases, were included in this group. Peripheral blood samples were taken between 8 and 9 AM on the day before lymph node dissection.[6]

In the first nine patients included in this group, blood samples were also taken during surgery and between 8 and 9 AM on the day after surgery, to determine if any patients who were PCR negative before surgery could have become positive because of surgery.

High-Risk Disease-Free Patients

The aim was to determine if the PCR assay allowed prediction of short-term relapse (clinically or radiologically detectable within 6 months) in high-risk but disease-free patients with MM. Thirty-three patients who had undergone lymph node metastasis resection within the last year and who had a negative clinical and radiological staging at the time of PCR were included in this group. Peripheral blood samples were collected at 4-month intervals.

Patients With Distant Metastases

The aim was to determine whether the presence of circulating melanocytes was correlated with the rapidity and severity of disease progression in patients with disseminated MM. Thirty-two subjects with visceral and/or skin metastases (stage III) were included in this group. A complete staging with brain, chest, and abdominal computed tomographic scans was done at inclusion and was repeated every 4 months. In 17 patients, blood samples were collected before any systemic treatment. In 20 patients, the samples were collected 1 hour before a course of chemotherapy (at least 4 weeks after the previous course): dacarbazine (n=6), fotemustine (n=2), fotemustine-dacarbazine (n=1), cysplatin-fotemustine-dacarbazine (n=7), interleukin-2–cisplatin (n=2), campthotecin 11 (n=1), or miltefosine (n=1). In 12 patients, samples were also obtained 1 hour after chemotherapy, to evaluate the immediate effect of the treatment on circulating melanocytes. The disease course in the 4 months following the PCR test was classified by a physician who was unaware of the PCR results. The tumor burden was considered as the sum of the volume of each metastasis deduced from bidimensional measures on computed tomographic scans and the results of clinical examinations. "Rapid" progression was defined as a twofold or greater increase in the tumor burden or the number of metastases within a 4-month period "stable" evolution, as less than a 25% increase in the tumor burden and no additional metastases within a 4-month period; and "slow" progression, as a greater than 25% but less than twofold increase in the tumor burden and number of metastases within a 4-month period.

Patients After Primary Tumor Resection

The aim was to determine if the PCR could detect residual disease in apparently healthy patients after the resection of primary melanoma. Ten patients with stage I disease with a Breslow level greater than 2 mm were included in this group.

immediately after surgery, none of whom was PCR negative before surgery became positive during or immediately after resection. Conversely, two patients who were PCR positive prior to surgery turned negative.

HIGH-RISK DISEASE-FREE PATIENTS

As shown in **Table 1**, 58 samples were obtained in 33 radiologically and clinically disease-free but high-risk patients. After a positive PCR test, a patient had a 3.8 times higher risk (relative risk [RR], 3.82; 95% confidence interval [CI], 58 to 9.22) to develop metastasis within the next 6 months than after a negative PCR test (χ^2, P=.002). After adjustment for thickness and site of primary melanoma (ie, axial vs peripheral) the RR was still high (RR, 5.14; CI, 1.04 to 27.4 and RR, 5.15; CI, 1.17 to 22.7, respectively).

PATIENTS WITH DISTANT METASTASES

Ninety-seven samples were collected in 32 patients with metastatic MM (**Figure 2**). Seventy-three of these 97 samples were obtained before all systemic treatment (n=23) or at least 4 weeks after a course of chemotherapy (n=50). Sixteen patients were positive on the first sample, and 20 on at least one sample collected at a

Figure 1. *Detection of tyrosinase transcripts in nine patients with stage II before, during, and after lymph node dissection. Ethidium bromide gel showing the presence of tyrosinase transcripts in blood samples collected before (B), during (D), or after (A) surgery. Numbers 1 through 10 indicate case numbers. The first nine subjects correspond to the first nine in Table 1, but the 10th subject, whose lymph nodes were shown to be free of metastases, is included as a control. Two rounds of amplification were performed with outer and nested primers of the tyrosinase gene. Base-pair (bp) size of the polymerase chain reaction product (210 bp) and of molecular-weight marker are indicated by arrowhead and arrows, respectively.*

Table 1. Relationship Between PCR Findings and Relapse in Disease-Free High-Risk Patients*

Group	Metastasis Within 6 mo After Test		
	PCR+	PCR−	Total
No metastasis	6	37	43
Metastasis (death within 6 mo)	8 (1)	7 (1)	15 (2)
Total	14	44	58

**Repetitive polymerase chain reaction (PCR) tests (n=58) were performed at a 4-month interval in 33 patients with melanoma considered clinically and radiologically disease free after node metastasis resections. Each PCR test was used to predict the course within the 6 months after the test, independently of the results of the previous tests, and a patient with a recurrence of disease after a test was no more tested. P<.002. Plus sign indicates positive; minus sign, negative.*

4-month interval during follow-up. These 73 samples were used to determine whether or not PCR can be predictive of rapidity and severity of progression within the 4-month interval following the test. As shown in **Table 2**, after a positive test result, rapid progression was four times more likely than slow progression or stable disease (RR, 4.11; CI, 1.93 to 8.76; χ^2, P=.0002). It is interesting to note that PCR was more often positive (41%) (**Table 3**) in patients with visceral metastases at the time of the test than in patients with only skin or lymph node metastases (7%) (χ^2, P=.012). After adjustment for the site of metastases (lymph node and skin only vs visceral), the RR for rapid progression was still high (RR, 3.99; CI, 1.70 to 9.39; χ^2, P<.0009). Fourteen of the 93 samples were collected immediately after a course of chemotherapy in 12 patients. The PCR was negative in 11 of these 12 patients.

PATIENTS WITH STAGE I DISEASE AFTER PRIMARY TUMOR RESECTION

The PCR test was positive in two of the 10 stage I melanomas after their resection. These two PCR-positive patients remained free of metastases after a 23-month follow-up, whereas two patients with negative test results developed metastases 13 and 6 months later, respectively.

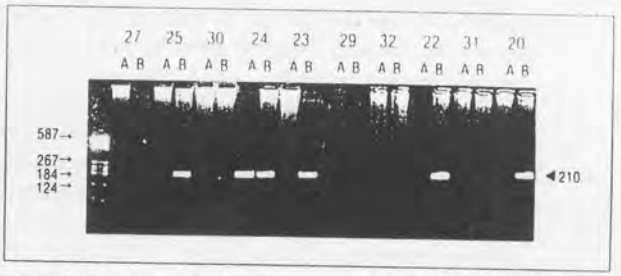

Figure 2. *Detection of tyrosinase transcripts in 20 of the blood samples collected before (B) and after (A) chemotherapy in patients with distant metastases (stage III). The complementary DNAs were independently amplified by the polymerase chain reaction with outer and nested tyrosinase primers. Aliquots of amplified samples were run in 1.5% agarose gel stained with ethidium bromide. Base-pair (bp) size of the polymerase chain reaction product (210 bp) and of molecular-weight marker are indicated by arrowhead and arrows, respectively.*

Table 2. Relationship Between PCR Findings and Disease Progression in Patients With Melanoma With Distant Metastases*

Evolution Within 4 mo After Test	No. (%)		
	PCR+	PCR−	Total
Rapid progression†	15 (60)	7 (14)	22
Slow progression†	5 (20)	20 (42)	25
Stable disease†	5 (20)	21 (44)	26
Total	25 (100)	48 (100)	73

**Seventy-three polymerase chain reaction tests performed in 32 patients with stage III disease: 23 samples collected before all systemic treatment, and 50 samples collected at a 4-month interval, at least 4 weeks after the last course of chemotherapy. Plus sign indicates positive; minus sign, negative.*

†See definitions in the "Materials and Methods" section. Progression (rapid or slow) vs stable, P=.05; relative risk, 1.42 (confidence interval 1.04-1.95); rapid progression vs stable or slow progression, P=.0002; relative risk, 4.11 (confidence interval, 1.93-8.76); and slow progression vs stable, not significant.

Table 3. Relationship Between PCR Findings in Patients With Melanoma With Distant Metastases and the Location of Metastases at the Time of the Test

Topography of Metastasis	No. (%)		
	PCR+	PCR−	Total
Visceral; brain, liver, and lungs	24 (41)	34 (59)	58 (100)
Skin or lymph nodes only	1 (7)	14 (93)	15 (100)
Total	25	48	73

**Seventy-three polymerase chain reaction (PCR) tests performed in 32 patients with stage III disease: 23 samples collected before all systemic treatment, and 50 samples collected at a 4-month interval, at least 4 weeks after the last course of chemotherapy. P=.012. Plus sign indicates positive; minus sign, negative.*

COMMENT

This is the first prospective study attempting to correlate PCR detection of circulating tumor cells with the outcome of patients with MM. Our findings in 93 patients confirm the previous data[3,4] showing that PCR was positive in a large proportion of patients with stage III MM with distant metastases (50% [16/32] in the first blood sample). The PCR was also often positive in stage II patients, and as one would

expect, more frequently before lymph node resection (44% [8/18 samples]) than after node resection (24% [14/58 samples], Table 1). Conversely, melanocytes were not found in the peripheral blood of normal subjects or in patients with non-MM metastatic cancers in the present study as well as in the study by Brossart et al.[4] This suggests that a positive PCR test result is specific for patients with melanoma and that venipuncture does not usually result in any contamination by normal melanocytes from the skin. Our findings also confirmed[4] that the PCR is sometimes positive in primary melanoma (stage I) after resection (two of 10), which can be interpreted as a proof of residual disease in apparently disease-free patients.

Although further study will be needed, our results suggest that PCR may have several useful clinical applications. The first would be the prediction of relapse after surgery in patients with stage II disease who were scheduled for lymph node surgery. Our results show that the presence of circulating melanocytes before lymph node dissection is a risk factor for short-term recurrence. If this finding is confirmed, the PCR could be useful in differentiating between patients with stage II who will benefit from lymph node dissection and those who probably will relapse very rapidly and in whom systemic therapy may be proposed as a preferred form of treatment. This is important since no current laboratory or radiological test assists in selecting among the various treatment modalities, ie, lymph node dissection or isolated limb perfusion vs systemic therapy. It is also noteworthy that our PCR data obtained before, during, and after surgery provided no evidence that lymph node resection releases tumor cells into the circulation. Obviously, this cannot be ruled out due to the relatively low number of patients. However, this fact has to be underlined because previous reports have suggested that surgical trauma may trigger the production of metastases.[7]

Another application for the PCR test described herein could be in the surveillance of disease-free high-risk patients with MM (Table 1). When a PCR test result is positive, the risk of relapse within the following 6 months is four to five times higher than when the test result is negative, even after adjustment for other risk factors. Although it is not very sensitive (53%), the PCR test can predict short-term recurrence with a good specificity (86%). If these results are confirmed, periodic PCR testing could be proposed as a tool for surveillance of high-risk patients.

A third prognostic application is the prediction of severity of evolution in patients with stage III disease. The course of metastatic disease varies widely among patients and the rapidity of the process cannot be predicted. Interestingly, our results indicate that the presence of circulating melanocytes is a risk marker for rapid and severe progression in patients with distant metastases. Although this marker is not highly specific (80%) and sensitive (68%), it would be possible to use the PCR for classifying patients with stage III disease into more homogeneous prognostic groups to evaluate the efficiency of future therapeutic strategies.

It is also noteworthy that the PCR was more frequently positive when the metastases involved the liver, lung, and brain compared with lymph nodes or skin. The former organs can be considered as prime candidates for blood-mediated metastases. Another interesting observation was the absence of circulating tumor cells in 11 of 12 patients who were tested immediately after a course of chemotherapy. This suggests that chemotherapy does not release living cells from the tumor and, moreover, that it may eliminate, at least transiently, circulating tumor cells.

A possible limitation of the PCR detection of circulating tumor cells in blood is that only one sample was collected on a given day. Thus, a discontinuous phenomenon such as transient release of neoplastic cells in blood may have been missed. This could account for the fact that, in this series, circulating cells were found in only 50% (16/32) of the first samples collected from patients with stage III MM. Therefore, the sensitivity of the method may be enhanced by pooling several samples collected at different times on the same day.

From a theoretical standpoint, it must be kept in mind that PCR detects melanocytes regardless of their metastatic potential. Indeed, most circulating tumor cells are thought to die rapidly, and only a few cells are able to complete the multistep process of selection leading to metastasis.[8,9] Nevertheless, it seems relevant to assume that a high rate of neoplastic cells released in the bloodstream might increase the probability of distant seeding. The prognostic correlations described herein support this assumption.

Finally, our results suggest that PCR detection of circulating melanocytes can predict the course of patients with stage II and III melanoma within the months following the test. The PCR tests repeated at a 6-month intervals could thus be useful in the clinical management of these patients. Whether the PCR could also predict the course within the following years and survival is an open question.

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Br. J. Cancer (1994), 70, 1224-1228

*NM*23 expression in metastasis of malignant melanoma is a predictive prognostic parameter correlated with survival

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Summary The management of patients presenting with metastatic malignant melanoma (MM) is hampered by the substantial variability in survival of these patients and the lack of prognostic markers. In the search for a reliable predictive parameter, we have investigated the expression of the *nm*23 gene, considered to be a major regulator of the metastatic process. We have analysed by Northern blot the *nm*23 mRNA level in tumour tissue obtained from metastases of 20 stage II and ten stage III patients with MM. Normal human tissues and benign naevi were simultaneously examined. The level of *nm*23 expression was highly heterogeneous in MM metastases, with a mean value which was higher than the mean level in normal tissues and naevi. Correlative study was focused on the overall survival following resection of the metastasis in which *nm*23 Northern blot analysis was performed. Patients displaying higher *nm*23 expression in metastatic tissue (above the mean level) tended to have a longer survival than others ($P = 0.08$), and this difference was significant for patients presenting with isolated regional lymph node involvement ($P = 0.035$). The time from biopsy of the primary MM to the appearance of the first lymph node metastasis also showed a positive correlation with the *nm*23 mRNA level in this metastasis. The present study is not only in accordance with previous reports showing that the *nm*23 gene may be implicated in MM progression, but also suggests the reliable value of *nm*23 expression as a prognostic marker for patients presenting with metastatic MM.

Current methods to identify the aggressive potential of malignant melanoma (MM) are limited. Even after occurrence of regional lymph node metastasis, patients may either pursue an indolent clinical course or rapidly die. The search for reliable prognostic parameters therefore appears vitally important in order to ensure adequate therapy, especially for advanced MM stages which are candidates for non-surgical treatment.

The production of clinically relevant metastasis is triggered by a complex series of linked sequential steps, some being genetically regulated by transient or permanent alterations at the DNA or mRNA level. The *nm*23 gene is thought to play a major role in this network of triggering signals (Rosengard *et al.*, 1989; Leone *et al.*, 1991). This gene was identified by differential colony hybridisation between related low- and high-metastatic murine k-1735 melanoma cell lines, a tumour system which contains clonal populations with qualitative differences in metastatic capacity in syngenic mice (Steeg *et al.*, 1988). mRNA levels of the *nm*23-1 gene were found to be approximately 10-fold higher in low-metastatic potential clones than in highly metastatic clones (Steeg *et al.*, 1988).

In human tumours, contradictory results were reported on *nm*23 gene expression. Reduced expression was found in primary, infiltrating ductal breast carcinomas with metastases in regional lymph nodes present at diagnosis (Bevilacqua *et al.*, 1989; Hennessy *et al.*, 1991). Low *nm*23 expression in breast tumours also correlated with decreased survival (Barnes *et al.*, 1991). These findings, however, cannot be generalised since low *nm*23 expression does not clearly imply poor prognosis in other types of human tumours such as colorectal carcinoma or neuroblastoma (Cohn *et al.*, 1991; Hailat *et al.*, 1991; Haut *et al.*, 1991). The prognostic value of the *nm*23 gene transcriptional activity in MM is suggested by the fact that this gene was originally cloned from murine melanoma cells, and also by some preliminary observations in human MM (Florenes *et al.*, 1992). In this report, we have tried to investigate the significance of *nm*23 expression as a parameter for the practical management of advanced-stage MM.

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Materials and methods

Tumour sampling

Tumoral tissue samples from 30 patients with MM were obtained through surgery. These patients were classified as stage II (regional lymph node involvement, $n = 20$) or stage III (distant lymph node involvement or visceral metastasis, $n = 10$). The histopathological characteristics of the primary cutaneous MM are detailed in Table I.

Each biopsy specimen was histologically identified as metastasis of MM involving lymph node in 25 cases, skin in four cases and liver on one case. A part of each fresh sample was stored in liquid nitorgen.

In addition, eight samples of human normal tissues (liver, breast, prostate, lymph node, spleen and ovary) as well as three benign naevi were analysed.

Northern blot analysis

Total RNA was isolated from frozen tissues by the guanidinium thiocyanate–caesium chloride method as previously described (Maniatis *et al.*, 1982).

Integrity of each RNA sample was ensured by (i) electrophoresis of a 2 μg aliquot on denaturing agarose–formaldehyde gel; and (ii) reverse transcription and polymerase chain reaction (PCR) amplification of the human GAPDH gene, which is expressed in almost all types of tissues. Northern blots were performed by running 10 μg of RNA on denaturing gels and transferring onto Hybond nylon membranes as indicated by the manufacturer (Amersham, UK).

The filters were UV cross-linked and hybridised to the *nm*23-H1 cDNA probe (a 900 bp *Bam*HI fragment from p*NM*23-H1 plasmid, kindly provided by Dr P.-S. Steeg, NCI, Bethesda, MD, USA). Filters were then stripped and rehybridised to a cDNA probe specific for human GAPDH to correct for the unequal amount of RNA loaded in each lane. The level of *nm*23 mRNA was adjusted relative to the amount of GAPDH RNA after densitometric scanning of the autoradiograms. GAPDH was chosen as an internal standard because this gene is refractory to transcriptional induction by various agents and is known to show a relatively constant expression among most tissues (Bosma & Kooistra, 1991; Zentella *et al.*, 1991).

Statistical analysis

Clinical and follow-up data were available in all patients and attempts were made to correlate *nm23* expression with prognosis.

Statistical evaluation was performed by BMDP package program. The proportion surviving was estimated by Kaplan–Meier method and compared by Mantel–Cox test.

Results

nm*23 expression*

The level of *nm23* expression was expressed as a percentage of the GAPDH mRNA level.

The mean level of *nm23* expression in eight normal tissues samples, i.e. liver, breast, prostate, lymph node, spleen and ovary (65%) was approximately similar to the mean *nm23* level in three benign naevi.

In the group with MM, expression was highly heterogeneous, ranging from 7% to 240% (Figure 1; Tables I and II).

Clinical correlations

A summary of statistical data is given in Table III.

Mean overall survival following metastasis resection was 21.6 months among the whole population of 30 patients. Within this population, patients with *nm23* RNA content above the mean level of *nm* expression (46.9%) tended to do better than others: $P = 0.08$ (Figure 2 and Table II). Furthermore, among the 20 patients presenting with only regional lymph nodes (stage II) at the time of Northern blot analysis, there was a significant correlation between *nm23* RNA level in the metastatic lymph node and the overall survival taking the node resection as a starting point. Indeed, stage II patients displaying *nm23* expression above the mean level had a longer survival than others: $P = 0.035$ (Figure 3 and Table II).

Unlike the overall survival, the disease-free interval (from resection of the analysed metastasis to the occurrence of relapse) was not significantly different among stage II patients with *nm23* expression above or below the mean level: $P = 0.48$ (Figure 4).

When the time of primary tumour resection was chosen as a starting point, a significant positive relation was observed between the time interval until the occurrence of the first metastasis and the *nm23* level in this metastasis. Indeed, among the subgroup of patients who had presented initially as stage I (isolated cutaneous tumour) and evaluated for *nm23* level in the first known metastasis ($n = 15$), the disease progression was slower in patients with *nm23* above the median level (28%): $P = 0.04$ (Figure 5 and Table II). The median *nm23* level was chosen as reference in this subgroup because almost all patients were above the mean level.

In addition, it must be noted that, at the time of lymph node metastasis resection, patients presenting with more disseminated disease (lymph node metastasis associated with involvement of other organs including skin) expressed lower *nm23* levels (mean 31%) than patients harbouring a single lymph node metastasis (mean 51%), but the difference was not significant.

There was no significant correlation between *nm23* expression and histological typing of primary cutaneous MM (Table I).

Table I Correlations between *nm23* expression in metastasis and histopathological characteristics of primary melanoma

Cases	*nm23 expression*[a]	*Histological type*	*Clark*	*Breslow (MM)*
1	7	NM	IV	4
2	10	NM	IV	1.5
3	11	SSM	IV	2
4	14	ALM	III	1.5
5	14	ALM	III	1.4
6	16	SSM	IV	2.7
7	17	ALM	V	5
8	20	SSM	II	0.6
9	22	SSM	II	0.8
10	22	SSM	III	1.4
11	22	SSM	IV	2.5
12	25	SSM	III	1.95
13	25	NM	IV	2.0
14	26	NM	II	0.9
15	28	ALM	IV	3.3
16	28	NM	III	2.4
17	29	ALM	IV	3.6
18	31	SSM	III	1.4
19	35	SSM	IV	1
20	41	SSM	III	1.4
21	46	SSM	IV	1.6
22	47	ALM	III	1.5
23	49	SSM	III	1.1
24	52	SSM	IV	2.5
25	63	NM	III	1.4
26	78	NM	IV	6
27	81	SSM	IV	5.8
28	88	Unclassified	V	14
29	218	Primary tumour unknown		
30	240	NM	III	1.4

[a]Analysed on early or late metastasis.

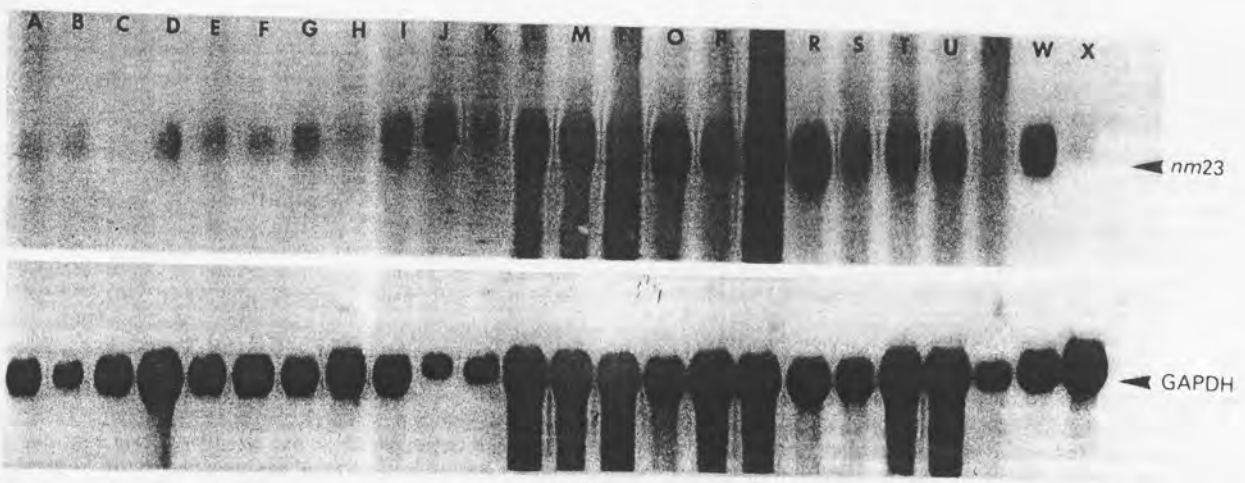

Figure 1 Northern blot analysis showing the *nm23* mRNA level in normal tissues, benign naevi and metastases of melanoma. Total RNA was hybridised to the 900 bp *Bam*HI fragment of *nm23*-H1 cDNA (top) and as a control to a GAPDH probe (bottom). Lanes A–C, normal tissues from liver, breast and prostate; lanes D–F, benign naevi; lanes G–X, metastases of melanoma.

Table II Correlations between *nm*23 expression, overall survival from the time of *nm*23 analysis, disease-free interval from the time of primary tumour resection and clinical staging

Cases	*nm23 expression*[a]	*Overall survival (months)*[b]	*Interval from primary tumour*[c]	*Clinical staging*[d]
1	7 (<m)	16		II
2	10 (<m)	7		II
3	11 (<m)	7		II
4	14 (<m)	4	16	III
5	14 (<m)	8		II
6	16 (<m)	6		III
7	17 (<m)	2		II
8	20 (<m)	9		III
9	22 (<m)	11	46	II
10	22 (<m)	29		III
11	22 (<m)	17	11	II
12	25 (<m)	11	20	II
13	25 (<m)	9		III
14	26 (<m)	3	21	III
15	28 (<m)	2		II
16	28 (<m)	20		III
17	29 (<m)	20	29	II
18	31 (<m)	4	35	II
19	35 (<m)	4	26	II
20	41 (<m)	10	34	II
21	46 (<m)	5	60	II
22	47 (>m)	15	32	II
23	49 (>m)	9	54	III
24	52 (>m)	5		III
25	63 (>m)	12	1	II
26	78 (>m)	14		III
27	81 (>m)	8		II
28	88 (>m)	33		II
29	218 (>m)	10		II
30	240 (>m)	22	11	II

[a]Analysed on early or late metastasis. (<m) and (>m) refer to the mean level of *nm*23 expression (46.9%), calculated in the whole population of 30 samples. [b]From the time of metastasis resection, i.e. from *nm*23 Northern blot anaysis. [c]Disease-free interval from resection of the primary cutaneous MM until occurrence of the first metastasis (restricted to 15 patients who had presented initially without metastasis and for whom *nm*23 analysis could be performed on the first metastasis). [d]Stage II, regional lymph node metastasis; stage III; visceral or disseminated metastases.

Table III Summary of statistical correlations between *nm*23 expression and patients' outcome

Correlation between NM23 expression and	*P-value (Mantel–Cox)*
Overall survival from metastasis resection among all patients	0.08
Overall survival from metastasis resection among stage II patients	0.035
Disease-free survival from metastasis resection among stage II patients	0.48
Time interval from primary MM resection to first metastasis among stage I patients	0.04

Discussion

Recent evidence indicates that the human *nm*23-H1 gene is located in 17q21.3, a chromosomal region known to contain the locus for early-onset familial breast–ovarian cancer and other genes involved in tumorigenesis (Steeg *et al.*, 1988; Leone *et al.*, 1991). This gene encodes one subunit of the enzyme NDP kinase (Gilles *et al.*, 1991) and is structurally related to the human *nm*23-H2 gene encoding a second subunit of NDP kinase and co-localising with *nm*23-H1 in this region (Stahl *et al.*, 1991). *nm*23 genes have also substantial homology with the predicted product of the *Drosophila melanogaster* developmental gene for abnormal wing discs (*awd*), which shows NDP kinase activity (Biggs *et al.*, 1990).

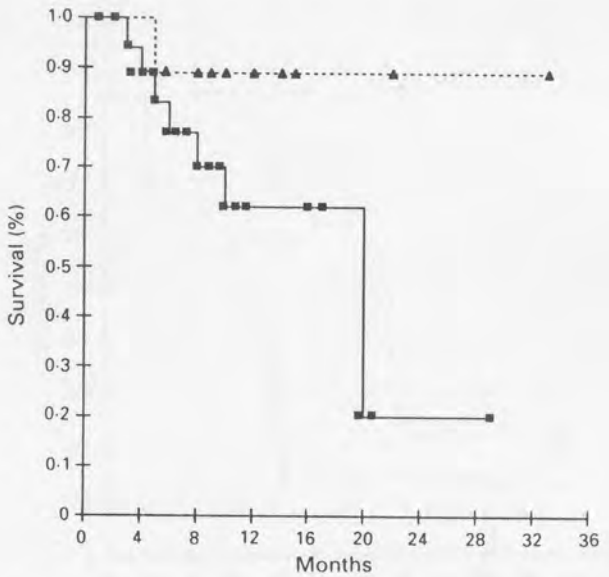

Figure 2 Kaplan–Meier graph showing the relationship between *nm*23 level in metastasis and overall survival following metastasis resection among the whole patient population, regardless of staging. The 30 cases were divided into two groups according to respective *nm*23 level compared with the mean *nm*23 expression (46.9%). Patients with *nm*23 expression above the mean level tended to do better (▲) than others (■), but the difference was not significant ($P = 0.08$).

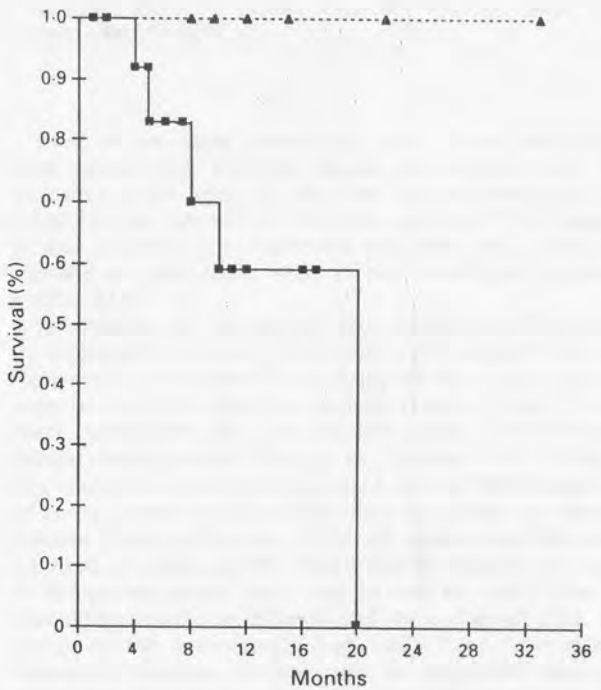

Figure 3 Kaplan–Meier graph showing the relationship between *nm*23 level in lymph node metastasis and overall survival following metastasis resection in stage II patients. The 20 cases were divided into two groups according to *nm*23 expression compared with the mean *nm*23 level (46.9%). Significantly longer survival was observed among patients with *nm*23 expression above the mean level (▲), when compared with others (■) ($P = 0.035$).

It has been postulated that NDP kinase may participate in signal transduction through G-proteins (Stryer, 1986).

Although demonstration has been provided that the *nm*23 gene may act as a metastasis-suppressor gene in at least some experimental models (Henderson, 1993), the role of *nm*23 is still unclear in human cancer. Attempts to use tumour levels of *nm*23 expression as a predictive marker have given rise to contradictory findings.

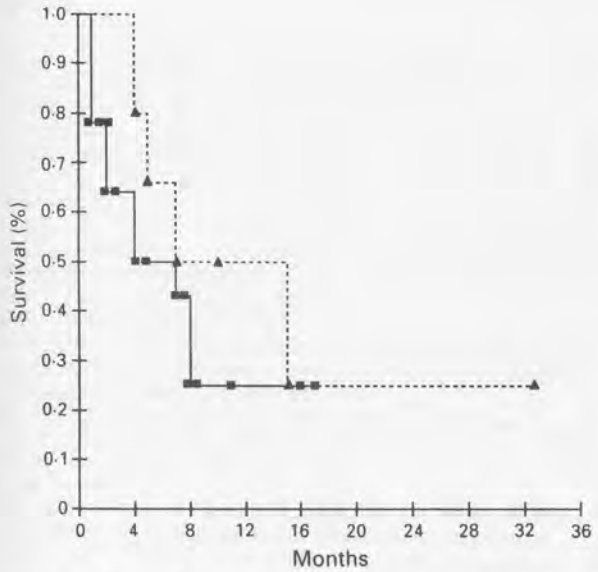

Figure 4 Kaplan–Meier graph showing the relationship between *nm*23 level in lymph node metastasis and disease-free survival following metastasis resection in stage II patients. Correlations were analysed in a way similar to Figure 3. There was no significant difference in survival ($P = 0.48$).

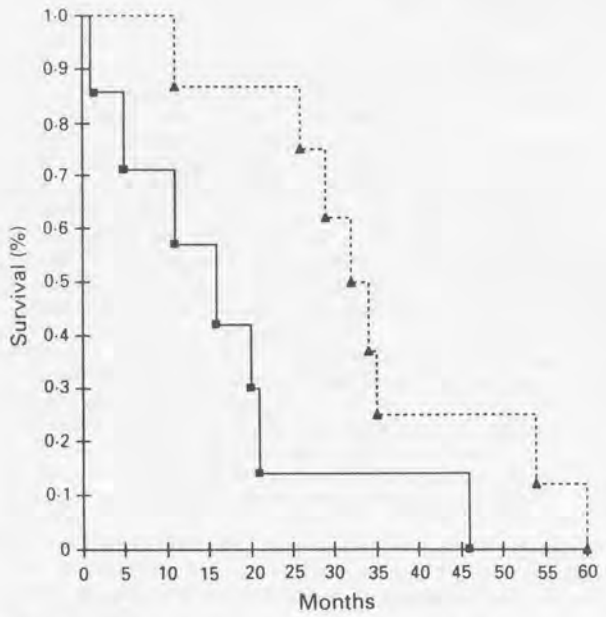

Figure 5 Relationship between *nm*23 levels in the first metastasis and the disease-free interval from resection of the primary cutaneous MM to the occurrence of metastasis. This graph is restricted to 15 patients who had presented initially without metastasis and for whom *nm*23 analysis could be performed on the first metastasis. Patients were divided into two groups according to *nm*23 expression compared with the median *nm*23 level (28%). Longer intervals were observed among patients with *nm*23 expression above the median level (▲), when compared with others (■): $P = 0.04$.

In some breast tumours, evidence suggesting that low *nm*23 mRNA levels may indicate a poor prognosis could be demonstrated, based on the fact that patients whose tumours showed reduced *nm*23-H1 expression had a higher rate of lymph node metastasis and reduced survival (Bevilacqua *et al.*, 1989; Hennessy *et al.*, 1991; Barnes *et al.*, 1991). In colorectal carcinoma however, *nm*23 expression correlated only with the occurrence of liver metastasis but not with lymph node involvement (Haut *et al.*, 1991; Yamagushi *et al.*, 1993). In addition, human colon carcinomas were found to exhibit enhanced *nm*23 mRNA expression compared with normal mucosa (Yamagushi *et al.*, 1993). Moreover, increased *nm*23 protein levels were observed, surprisingly, in advanced-stage neuroblastoma (Hailat *et al.*, 1991).

A recent report has suggested that expression of the *nm*23 gene may be related to rapid progression in patients with MM. Florenes *et al.* (1992) observed that the *nm*23 mRNA level tended to be higher in secondary tumours occurring after prolonged relapse-free interval from primary diagnosis. Nonetheless, this study was only retrospective and did not attempt to show the usefulness of *nm*23 expression as a predictive parameter of prognosis.

The prognosis of patients with advanced MM actually remains poorly defined, since substantial variability in survival can be observed. In patients with regional nodal disease (stage II), the likelihood of systemic recurrence has been only correlated with the size and number of involved nodes, capsular effraction and more recently with some biological parameters (Sirott *et al.*, 1993).

In the present report, we have tried to investigate the significance of *nm*23 expression as a prognostic marker for MM patients who have developed metastasis (stage II or III). We have therefore focused our study on the link between this expression and the time from biopsy of metastasis to the death of the patient (overall survival).

Our results proved to be of particular interest with regard to patients presenting with regional node invasion (stage II) at the time of Northern blot analysis. Among this subgroup, overall survival following metastasis resection was indeed significantly longer for patients with *nm*23 expression in metastasis above the mean level. These data are not only in accordance with a putative relationship between *nm*23 transcriptional level and progression of the disease, as suggested by Florenes *et al.* (1992), but they also provide the additional interest to be potentially helpful for the therapeutic strategy.

From a theoretical standpoint, some of our findings also seem noteworthy, although devoid of practical value. The fact that *nm*23 levels in the first known metastasis were related to the interval of time from primary MM diagnosis further supports the hypothesis that the *nm*23 gene may regulate at least some steps of the metastatic process in human MM.

Nonetheless, the mechanism by which the *nm*23 gene may be implicated in tumour progression still remains far from clear since, in contrast to what should have been expected, some of our MM metastasis samples exhibited higher level of *nm*23 expression than benign naevi and normal tissues. Similar findings were reported by Florenes *et al.* (1992). In this context, it must also be pointed out that *nm*23 expression in colon cancer can be higher than in normal surrounding mucosa (Yamagushi *et al.*, 1993). An explanation for the low amounts of *nm*23 product which can be observed in normal or benign neoplastic tissue may be that the *nm*23 gene may play different roles in differentiated the malignant cells. With regard to the unexpectedly high *nm*23 RNA level in some aggressive tumours, it may also be suggested that *nm*23 molecular alterations, other than reduced expression, may result in aggressive tumoral behaviour. This hypothesis appears relevant in at least some cases of aggressive neuroblastoma harbouring *nm*23 genomic amplification and mutation (Hailat *et al.*, 1991).

In conclusion, the present study suggests the prognostic value of *nm*23 expression in the practical management and therapeutic strategy of MM patients and should now be confirmed by larger series and clinical trials.

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Expression of *FGF1* and *FGFR1* in human melanoma tissues

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Fibroblast growth factor 2 (FGF2) has been implicated in the pathogenesis of malignant melanoma (MM), but the role of other FGFs and their receptors (FGFRs) is not elucidated. To determine whether FGF1 and FGFR1 may be involved in MM growth *in vivo*, we have studied the expression of the *FGF1* and *FGFR1* genes in 77 fresh MM biopsy samples, using RT-PCR analysis. Samples of benign nevi, normal skin and carcinoma cell lines were included as controls. Using RT-PCR analysis, expression of *FGF1* and *FGFR1* was observed in 69/77 and 68/77 cases, respectively. Immunohistochemical detection of the FGFR1 protein was positive in reactive stromal cells and at a much lower level in neoplastic cells. *In situ* hybridization experiments demonstrated *FGFR1* mRNA mainly located in the stromal component. Southern blot analysis of genomic DNA prepared from MM tumors did not show any structural alteration of the *FGFR1* gene. There was no correlation between *FGF1/FGFR1* expression and the usual clinicopathological parameters of MM. We conclude that *FGF1* and *FGFR1* are frequently co-expressed in MM, a situation that may contribute to aberrant autocrine and paracrine pathways. Due to the absence of correlation with clinico-pathological parameters, this expression cannot be used as a marker of prognosis in the management of MM patients.

Key words: FGF1, FGFR1, melanoma.

Introduction

Fibroblast growth factors (FGFs) are a family of structurally related polypeptides including nine members known to date: acidic FGF/FGF1, basic FGF/FGF2, int2/FGF3, hst/kaposi FGF/FGF4, FGF5, FGF6, KGF/FGF7, FGF8 and FGF9 (reviewed in refs 1 and 2). These molecules have angiogenic and neurotrophic properties, are mitogenic for a wide variety of cells and play important roles in inductive events in development.[1] FGFs interact with high affinity tyrosine kinase receptors encoded by at least four distinct genes including *FGFR1/flg*, *FGFR2/bek*, *FGFR3* and *FGFR4* (reviewed in refs 3 and 4). The common structure of the receptors consists of an extracellular region containing three immunoglobulin (Ig)-like domains, a transmembrane domain and a cytoplasmic region comprising the tyrosine kinase domain. Multiple secreted and membrane-bound receptor isoforms with different ligand specificities can be generated due to alternative splicing events.[3] Alternative splicing also results in FGFRs containing either two or three Ig-like domains in the extracellular regions. This variability is subject to cell- and tissue-specificity.[3,5]

Evidence for FGF participation in human carcinogenesis has been documented in some tumoral models, especially Kaposi sarcoma,[6,7] mammary and prostatic carcinomas, in which FGFs play an important role in the interactions between malignant cells and their environment.[8–11] Furthermore, it has been demonstrated that the development and progression of human malignant melanoma (MM) is specially influenced by FGF2.[12–16] This molecule is produced by fibroblasts and epidermal keratinocytes but not by normal melanocytes, which require an exogenous source of FGF2 for growth in culture, a feature lost by cell lines derived from metastatic and primary invasive melanomas.[12–16] These cell lines, as well as *in vivo* metastatic and primary invasive melanomas, express FGF2 mRNA, whereas MM *in situ* and benign melanocyte nevi do not.[17] However, the study of FGF2 at the protein level yielded contrasting results since expression of this factor was not consistent in all MM tested.[18] Whether other members of the FGF family can mediate MM growth by acting as autocrine mediators of cell proliferation is not known. In this respect, characterization of FGFR status in MM and identification of possible FGF ligands is paramount to an exhaustive understanding

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of MM pathogenesis. *FGFR1* has been studied in MM cell lines and in MM samples, and the data tend to indicate that expression of *FGFR1* is required to sustain the proliferation of normal and neoplastic melanocytes and may prevent them from undergoing terminal differentiation.[19,20] In this study, we have analysed fresh MM biopsy samples for alterations in *FGF1* and *FGFR1* genes expression and structure, to evaluate their respective influence in an environment as close as possible to the *in vivo* conditions.

Materials and methods

Tissue samples and cell lines

A series of 77 MM biopsy samples, including 59 regional lymph node metastases, 13 cutaneous metastases, two liver metastases and three primary tumors, were obtained through surgical removal. In addition, a panel of 10 benign nevi and normal skin was studied simultaneously. A part of each sample was submitted to conventional histopathological processing and the other part was snap frozen in liquid nitrogen and stored at − 80°C until use. The presence of high amounts of neoplastic cells was checked on frozen sections prior to storage.

The MDA-MB-134 and the MDA-MB-231 mammary carcinoma cell lines (ATCC, Rockville, USA), which constitutively exhibit amplification and/or overexpression of the *FGFR1* and *FGF1* genes were used as controls.[9,21]

RT-PCR analysis of *FGF1* and *FGFR1* expression

Total RNA was extracted from frozen biopsy samples according to standard procedures.[22] Total RNA (2 mg) was used to prepare the first strand of cDNA using oligo(dT) primers and the AMV reverse transcriptase, according to the recommendations of the supplier (Promega-Biotec).

Specific primers for *FGF1* were 5′ GAA GCC CAA ACT CCT CTA CTG TAG C 3′ (sense) and 5′ TGT TGT AAT GGT TCT CCT CCA GC 3′ (antisense). It resulted in a PCR product of 259 bp (position 72–308). *FGFR1* primers were selected to amplify sequences encoding protein regions located in the extracellular domain, as described.[23] Three bands of 1098, 832 and 976 bp can be observed, which indicates the presence of transcripts corresponding to receptors with three Ig-like domains, two Ig-like domains and two Ig-like domains + insert, respectively.[21,23]

PCR cycle profile was denaturation at 94°C for 1 min (2 min for the first cycle), annealing at 56°C (FGF1) or 61°C (FGFR1) for 2 min and extension at 72°C for 3 min (10 min for the last cycle). The PCR products were separated on agarose gels, visualized by ethidium bromide, and then transferred into nylon membranes. PCR analyses were repeated at least twice on each sample. In each panel, the same relative differences in the amount of PCR products were reproduced, thereby indicating that the variations observed in the intensity of RT-PCR bands reflected differences in mRNA concentration and not random variability.

The specificity of PCR products was checked by hybridization with synthetic oligoprobes corresponding to the coding sequences of *FGF1* (5′ CAG CTG CAG CTC AGT GCG GAA AGC 3′; positions 210–233) and *FGFR1* (5′ ATA ACG GAC CTT GTA GCC TCC 3′; positions 610–630, located in the third exon). Integrity of each RNA sample was checked by amplification of the actin gene with specific primers.[21]

Southern blot analysis of the *FGFR1* gene

DNA was extracted from frozen tissue samples, digested with Eco RI restriction enzyme and transferred onto nylon membranes as described previously.[22] The *FGFR1* probe used for Southern blotting was the 340 bp Eco RI-Bgl II insert from the pOL10 plasmid, encoding a portion of the extracellular region of the receptor.[9]

In situ hybridization for localization of *FGFR1* RNA expression

In situ hybridization experiments were conducted as described previously.[10,24] Briefly, radiolabeled probes were generated after linearization of pOL10 plasmid DNA by restriction enzyme digestion and *in vitro* translation in the presence of ^{35}S-labeled UTP, in standard reactions using T3 or T7 RNA polymerase. Serial microtome frozen sections were hybridized with 50,000–75,000 cpu/μl ^{35}S-labeled sense or anti-sense cRNA probe. After the washes, the slides were dipped into Kodak NTB-2 nuclear track emulsion diluted 1 : 1 and autoradiographed for 1–3 weeks. After photographic development, the slides were stained with toluidine blue and analysed on a Zeiss Axiophot microscope.

Positive controls were two breast carcinoma samples which had been previously identified as strongly positive for *FGFR1* RNA.[10]

Immunohistochemistry for localization of *FGFR* protein products

Immunohistochemistry was performed on frozen sections using a three-step immunoperoxidase technique as previously described.[25] The primary antibodies used for *FGFR1* detection were (a) a mouse IgG1 (clone 19B2, Upstate Biotechnology Inc., Lake Placid, NY, USA) spe-

Table 1. RT-PCR analysis of FGF1 and FGFR1 expression in fresh tissue samples

Sample	Actin	FGF1	FGFR1
MM1	+ +	+ +	+ +
MM2	+ +	+ + +	+ +
MM3	+ +	+ + +	+ +
MM4	+ +	+ + +	+ +
MM5	+	–	+ + +
MM6	+ +	+ +	+ +
MM7	+ +	+ +	+ + +
MM8	+	–	+ +
MM9	+ +	+ +	+ +
MM10	+ +	–	–
MM11	+ +	+ + +	–
MM12	+ +	+ +	–
MM13	+ +	+ +	–
MM14	+ +	+ +	+ +
MM15	+ +	+	+ + +
MM16	+ +	+ +	+ + +
MM17	+ +	+ +	+ +
MM18	+ +	+ +	+ +
MM19	+	+ +	+ + +
MM20	+ +	+ +	+ + +
MM21	+ +	+	+ +
MM22	+ +	+ +	+ +
MM23	+ +	+ +	+ +
MM24	+ +	+ +	+ +
MM25	+ +	+ +	+ + +
MM26	+ +	+ +	+ +
MM27	+ +	+ +	+ +
MM28	+ +	+ +	+ +
MM29	+ +	–	+
MM30	+	+ +	+ +
MM31	+ +	+ + +	+
MM32	+ +	+ +	+ +
MM33	+ +	+ + +	+ +
MM34	+ +	+ +	+ +
MM35	+ +	+ + +	+
MM36	+ +	–	–
MM37	+ +	+	+
MM38	+ +	–	–
MM39	+ +	+ + +	+
MM40	+ +	+ +	+
MM41	+ +	+ + +	–
MM42	+ +	+ + +	+ +
MM43	+ +	+ +	+
MM44	+ +	+	+ + +
MM45	+ +	+ +	+
MM46	+ +	+ +	+
MM47	+ +	–	–
MM48	+ +	+ + +	+ +
MM49	+ +	+ + +	+ +
MM50	+ +	–	+
MM51	+ +	+ + +	+
MM52	+ +	+ +	+ +
MM53	+	+	–
MM54	+ +	+ +	+ + +
MM55	+ +	+ +	+ +
MM56	+ +	+ + +	+
MM57	+ +	+ +	+ +
MM58	+ +	+	+ +
MM59	+ +	+	–
MM60	+ +	+ + +	+ + +
MM61	+ +	+ +	+ + +
MM62	+ +	+	+ + +
MM63	+ +	+ +	+
MM64	+ +	+ +	+
MM65	+ +	+ +	+
MM66	+ +	+	+ +
MM67	+ +	+	+
MM68	+ +	+	+
MM69	+ +	+ +	+ +
MM70	+ +	+	+ +
MM71	+ +	+ + +	+
MM72	+ +	+ + +	+
MM73	+ +	+ + +	+
MM74	+ +	+ +	+
MM75	+ +	+ + +	+
MM76	+ +	+ + +	+
MM77	+ +	+	+ +
N1	+ +	+	+
N2	+ +	+ + +	+ + +
N3	+ +	+ +	+ + +
N4	+	+ +	+
N5	+	–	–
N6	+ +	–	–
N7	+ +	+ +	+ +
N8	+ +	+ +	+ + +
Normal skin 1	+ +	+	+
Normal skin 2	+ +	+	+ +
MDA 134	+ +	+ +	+ + +
MDA 231	+ +	+ + +	+ +

MM, malignant melanoma; N, benign nevus; MDA 134/231, breast carcinoma lines.

cific for the extracellular domain of both FGFR1 and FGFR2 and (b) a mouse IgM monoclonal anti-human FGFR1 (clone VBS1, Immunotech, Marseille, France). Various durations (1–12 h) of primary Mab incubation were performed at room temperature to increase the threshold level of sensitivity. Melanoma cells were simultaneously identified on serial sections using the HMB45 Mab (Immunotech, Marseille, France).

Statistical analysis

Statistical analysis of the correlations between *FGF1/FGFR1* expression and clinicopathological parameters was performed by Fisher and Log Rank tests.

Results

FGF1 and *FGFR1* transcripts can be detected by RT-PCR in most MM tumors

Using RT-PCR analysis, expression of *FGF1* or *FGFR1* was detected in 69/77 (90%) and 68/77 (88%) MM samples,

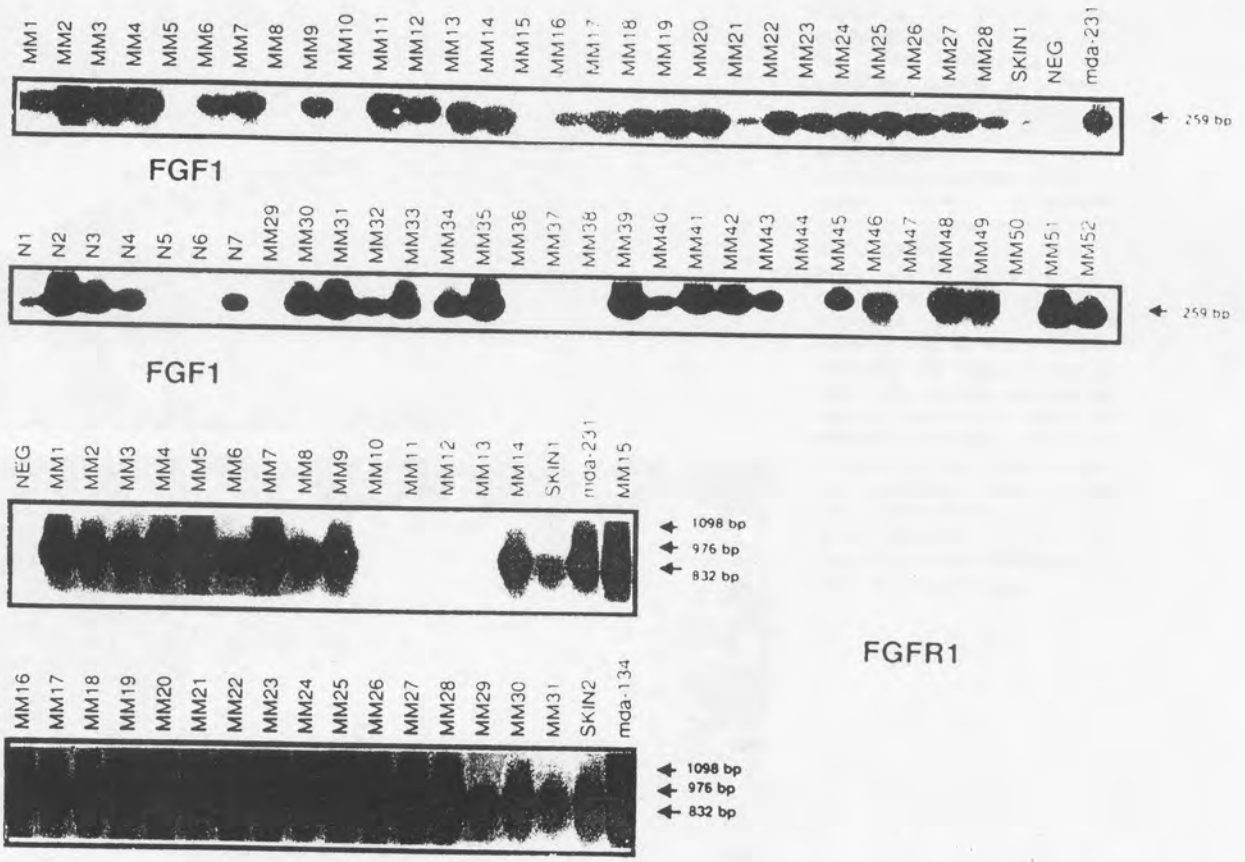

Figure 1. RT-PCR analysis of the expression of *FGF1* and *FGFR1* in fresh tissue samples of malignant melanoma (MM) and benign nevi (NN): numbering is the same as in Table 1. Each lane contained cDNA samples assayed for amplification with the indicated primers and hybridized by Southern blotting using internal oligonucleotidic probes, as described in Materials and methods. Arrows indicate molecular weights in base pairs. NEG, negative control (distilled water submitted to PCR); MDA-MB-134 and MDA-MB-231 breast carcinoma cell lines, positive control cell lines; SKIN 1 and 2, different specimens of normal skin.

respectively (Figure 1 and Table 1). Dissociation between *FGF1* and *FGFR1* expression was observed in nine tumors, which were negative for either *FGF1* (four cases) or *FGFR1* (five cases). Negativity for both *FGF1* and *FGFR1* was observed in four cases. Semi-quantitative estimation of RT-PCR signals (+ or + + +) according to the intensity of bands showed that there was no correlation between the respective levels of expression of *FGF1* and *FGFR1*, various combinations of positivities being observed (Table 1).

FGF1 and *FGFR1* are also expressed in most benign nevi and in normal skin

Normal skin showed consistent expression of both *FGF1* and *FGFR1* in the two analysed samples (Figure 1 and Table 1). All but two specimens of benign nevi were positive (Figure 1). In contrast with MM samples, there was no dissociation between *FGF1* and *FGFR1* expression in this group.

Immunodetection of FGFR1 protein products gives strong positivity in reactive stromal cells but weak staining in malignant cells

The 10 tumors analysed by *in situ* hybridization were next analysed by immunohistochemistry. Using a standard immunohistochemical detection (incubation time of 1 h at room temperature, dilution ratio of 1/100 for the primary MAb), FGFR1 protein products were detected only in stromal cells, i.e. fibroblasts, a few endothelial cells and histiocytes located in the connective walls within the tumor (Figure 2A, B). However, after prolonged incubation of the primary antibody (12 h at room temperature), it was possible to identify a faint membrane staining on neoplastic cells, suggesting a low level of protein expression. Positive immunodetection using the

Figure 2. Immunohistochemical localization of FGFR1 protein in melanoma. The specimen (MM 5) is a lymph node metastasis analysed on frozen sections. (A, B) FGFR1 immunodetection after standard incubation time of the primary FGFR1 monoclonal antibody, at low power view and high power view, respectively. FGFR1 protein product is only detected in stromal fibroblasts within connective walls (arrow), whereas melanoma cells are negative (empty star). (C) Control immunodetection performed with the HMB-45 antibody, which discloses a sinusal nest of positive melanoma cells (empty star) under the capsula (black dot). Residual lymphocytes (black star) and fibroblasts (arrow) are negative.

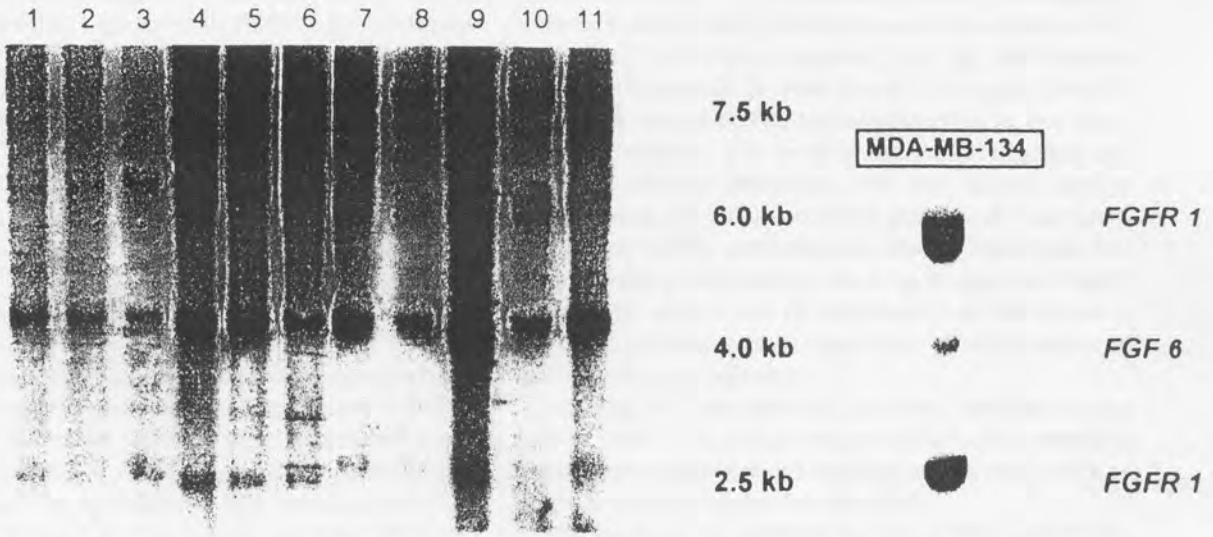

Figure 3. Southern blot analysis of the *FGFR1* gene in a representative panel of MM sample DNAs (MM1–11). Genomic DNA was extracted from frozen tissues; hybridization was carried out with *FGFR1* and *FGF6* probes, the latter acting as an internal control. Another internal standard was provided by an *Ankyrin* probe in lanes 4–11, which results in a 7.5 kb band. There was neither rearrangement nor amplification in the melanoma samples. Amplification of *FGFR1* in the mammary carcinoma cell line MDA-MB-134 is shown for comparison. Gene copy numbers were evaluated visually and by densitometer scanning.

control HMB-45 antibody was obtained in all cases (Figure 2C).

FGFR1 RNA is mainly produced by reactive cells, and at lower degree by neoplastic cells

To identify the site of expression of *FGFR1*, we selected a panel of 10 tumors displaying strong signals of *FGFR1* expression by RT-PCR. *In situ* hybridization using long exposure time (up to 20 days) revealed a faint signal located in both neoplastic and reactive components of MM tumors (data not shown).

The *FGFR1* gene does not show major structural alteration in MM

Southern blot analysis of DNA prepared from MM tumors detected two major bands of 6 and 2.5 kb after Eco RI digestion (Figure 3). A similar profile of restriction fragments was observed in samples from benign nevi and normal skin. There was no defect or additional band that could suggest any genomic rearrangement. Comparison of hybridization signals obtained with the *FGFR1* probe and with the *FGF6* control probe did not show any amplification of the *FGFR1* gene (Figure 3).

Absence of correlation between *FGF1/FGFR1* expression and follow-up data of MM

Using the Fisher test, there was no relationship between the different levels of *FGF1/FGFR1* expression as evidenced by RT-PCR (+ to + + +) and the Breslow staging of the primary tumors ($P=0.7$ for *FGF1*; $P=0.47$ for *FGFR1*). Using the Log Rank test, there was no statistically significant correlation between the different levels of *FGF1/FGFR1* expression as evidenced by RT-PCR (+ to + + +) and clinicopathological parameters including: (a) the overall survival after metastasis resection ($P=0.29$ for *FGF1*; $P=0.8$ for *FGFR1*), (b) the time from the analysed metastasis (in stage 2 patients) to the next relapse ($P=0.66$ for *FGF1*; $p=0.99$ for *FGFR1*), (c) the time from the primary tumor to the first metastasis ($P=0.09$ for *FGF1*; $P=0.58$ for *FGFR1*).

Discussion

There is now accumulating evidence that FGFs are important factors in the development and progression of MM.[12–16] In contrast to FGF2, little is known about the status of the FGF1 and FGFR1 molecules in MM. Studies by Becker *et al.*[19] and Mattei *et al.*[20] have found expression of the *FGFR1* gene in MM. This report showed that *FGFR1* was expressed in its different forms in normal and neoplastic melanocytes. *FGFR1* RNA expression was detected in all stages of melanocytic tumors and in normal melanocytes by Northern blotting and RT-PCR. The present study strengthens the data reported previously on the expression of the *FGFR1* gene in the vast majority of MM cases[19,20] and provides additional information about the cellular distribution of *FGFR1*. The latter is expressed by both stromal and neoplastic cellular components,

although at a lower degree in the latter case. This is noteworthy because high levels of *FGFR1* mRNA detected by RT-PCR could have simply resulted from increased numbers of endothelial cells which are known to express *FGFR1*.[26] The present series also confirms that the *FGFR1* gene is not structurally altered in MM. Moreover, it demonstrates *FGF1* mRNA expression in most MM samples, a result not reported so far.

Taken in conjunction with the proposed properties of FGF2 in MM,[12–16] our findings suggest the possibility of an autocrine loop of stimulation for MM growth, including FGFR1 as potential receptor and either FGF2 or FGF1 as potential ligands. Several *FGFR1* transcripts produce proteins which have similar affinity for FGF1 and FGF2.[3,4] The 2 and 3 Ig-domain isoforms of human FGFR1 seem equally responsive with respect to the affinity of FGF1 and FGF2 binding.[3] The specificity of FGF binding appears in fact to be determined by the carboxy terminal half of the third Ig-like domain of FGFR1.[3] The fact that a few tumors in our series were negative for FGF1 expression but positive for FGFR1 suggests that other members of the FGF family such as FGF2 and FGF4, which are known to bind FGFR1 with high affinity, can be involved in this putative autocrine loop. However, co-expression of a growth factor and its receptor, as evidenced herein, is a necessary condition for an autocrine loop but does not demonstrate it.

The observation that oligomers targeted against regions of the human *FGFR1* mRNA severely inhibit the proliferation of normal melanocytes[19] further supports the hypothesis of an FGF/FGFR1 autocrine loop. It is noteworthy, however, that a few tumors expressing FGF1 in our series were negative for *FGFR1* expression, thereby suggesting the possible involvement of other types of FGFRs which can bind FGF1 with high affinity.[1–3]

However, one must underline that some cell lines like MDA-MB-134, which overexpresses *FGFR1* mRNA, respond to FGF treatment with growth inhibition, in contrast to MCF-7, which responds to FGF stimulation by increased growth.[27] There is therefore difficulty in assigning mechanistic significance to co-expression of FGFR1 and FGF1 in MM tumors because the consequence of FGFR1 stimulation by FGF1 certainly depends upon features other than receptor–ligand affinity. These parameters may include ability of FGFR1 to form homodimers or heterodimers with other FGFRs, or with splice variants capable of acting in a dominant negative fashion, and the presence or absence of downstream effector molecules. Other crucial factors may be low affinity heparan sulfate proteoglycan binding sites, which are present both at the cell surface and in the extracellular matrix and are necessary for FGF1 activity.

It must be stressed also that FGFR1 expression in MM analysed herein was certainly weak at both RNA and protein levels because the signals observed in neoplastic cells by either *in situ* hybridization or immunohistochemistry were faint when compared with the MM reactive stromal component or with breast carcinoma controls. The weakness of FGFR1 immunodetection in our study certainly reflects a low level of expression that may not result in efficient interaction with the ligands. Among benign nevi, we did not observe dissociated expression of FGF1 or FGFR1, and it therefore may be speculated that escape from growth control resulting in aggressive behavior of MM tumors may be the result of an imbalance in the ligand–receptor ratio rather than overexpression of both ligand and receptor.

Although we can show by Southern blotting that the *FGFR1* gene is not subject to gross structural abnormality such as rearrangement or amplification, the occurrence of point mutations cannot be excluded.

Our study further supports the role of FGF1 and FGFR1 in human cancer in general, as suggested by previous reports. In tumor cells of pancreatic adenocarcinomas for instance, *FGF1* or *FGFR1* RNAs are often detected, with the presence of potential autocrine loops in 46% of cases.[28] In a significant number of tumors of the central nervous system, FGFR1 is expressed and associated with tumor progression.[29,30] In contrast with MM, FGFR1 synthesis in breast carcinoma is mainly located in neoplastic cells.[10] It appears that the profile of *FGF1/FGFR1* expression is not strictly similar depending upon the types of human tumors. One has now to characterize the cascade of events orchestrated by FGFR and their ligands as a prelude to the elaboration of new therapeutic strategies.

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