Table 2 Hormone values and clinical details of the ten patients with Yq microdeletions

Patients											
	1	2	3	4	5	6	7	8	9	10	11
Age (years)	45	44	43	42	35	36	36	55	46	34	48
Testicular volume (ml) right/left	5/8	8/10	4/3	17/15	7/7	17/16	14/13	18/11	10/9	8/7	5/5
Sperm count ($X10^6$/ml)	0	0	0	0.7	0.06	0	0.2	1.9	0	0	0
Deleted AZF regions	a,b,c	b,c	b,c	c	c	c	c	c	c	c	c
Inhibin B (pg/ml)											
Serum	<15	<15	<15	195	42	300	100	90	<15	<15	<15
Seminal plasma	<15	<15	<15	107	30	108	28	660	110	<15	<15
FSH (mIU/ml)	40.3	12.6	60.1	4.2	28.8	5.7	16.3	8.7	21.5	10.3	31.9
Histology	SCO	SCO	GA	GA	GA	HYPO			GA	GA	GA
Sperm recovery	–	–	–	+	+	+	+	+	+	–	–
Percentage of apoptotic cells (%)				2.5	5.0	4.0			7.5		7.0

SCO Sertoli cell-only, *GA* germ cell arrest, *HYPO* hypospermatogenesis

decline in the total sperm concentration from an average of 0.7 x 10^6 per ml to 0.02 x 10^6 per ml over 25 months. The serum and seminal plasma Inhibin B levels decreased from 195 pg/ml and 107 pg/ml to 35 pg/ml and 32 pg/ml, respectively. Patient 5 showed a decline in total sperm concentration from 0.06×10^6 per ml to azoospermia over 34 months. Serum and seminal plasma Inhibin B levels decreased from 42 pg/ml and 30 pg/ml to 18 pg/ml and 15 pg/ml, respectively.

Apoptosis was evaluated in the testes of 5 patients with AZFc deletions (patient 4, 5, 6, 9 and 11). Fifteen patients without AZFc deletions whose testicular histology were hypospermatogensis (3patients) or germ cell maturation arrest (12 patients) were also evaluated for apoptosis in testes. There was no significant difference in the testicular histology between these two groups.

The percentage of apoptotic germ cells in the testes of patients with AZFc deletions were significantly increased compared to those of patients without AZFc deletions and patients with obstructive azoospermia (5.2% vs. 2.1%, $p<0.01$; 5.2% vs. 1.0%, $p=0.01$; Table 3).

Table 3 Analysis of apoptosis in germ cells of testes

	Percentages of apoptotic cells (mean±SD)
Patients with AZFc deletions ($n=5$)	5.2±2.0[a,b]
Patients without AZFc deletions ($n=15$)	2.1±0.9
Obstructive asoospermic patients ($n=5$)	1.0±0.7

[a] Significantly different from patients without AZFc deletions ($P<0.01$)

[b] Significantly different from obstructive asoospermic patients ($P=0.01$)

Discussion

In this study, seven out of 117 (6.0%) patients with azoospermia and 4 out of 34 (11.8%) patients with severe oligozoospermia had Y chromosome microdeletions. These findings were consistent with previous reports of microdeletion frequencies between 6.2 and 25.9% in Japanese males [14, 15]. In the present study population, the frequency of Y chromosome microdeletions was lower in azoospermic patients than in oligozoospermic patients. Other Japanese studies [14] also reported a low frequency of Y chromosome microdeletions in azoospermic patients (4.2%) in comparison to oligozoospermic patients (15.9%). Nagata *et al.* [16] reported that the sperm retrieval rate by testicular sperm extraction in Japanese azoospermic patients was low in comparison to other studies. Other common genetic causes may exist in Japanese azoospermic patients. Eight out of 11 patients with Y chromosome microdeletions had complete AZFc deletions (b2/b4 deletion). The seminal phenotype of patients with complete AZFc deletions varied from azoospermia to severe oligozoospermia. Progressive regression of the germinal epithelium over a period of time has been reported which may be an explanation for such variable phenotypes [5]. However, Oates *et al.* [17] reported that 4 patients with AZFc deletions had stable sperm production over time. The discrepancies between the studies may have been due to the small number of patients.

In this study, 2 patients with AZFc deletions were followed over 2 years. Both patients exhibited a decline in total sperm concentration over 2 to 3 years, associated with a decrease in serum and seminal plasma Inhibin B levels. This finding supports a hypothesis of progressive depletion of the seminiferous epithelium. There is an association between serum Inhibin B levels and testicular pathology in

patients with AZFc deletions [18]. The current study also suggested that Inhibin B is a good marker for spermatogenic potential in patients with AZFc deletions. However, further studies with a greater number of study patients will be required to confirm the progressive decline of spermatogenic potential in patients with AZFc deletions and the utility of Inhibin B as a marker of spermatogenesis.

Mammalian spermatogenesis is a highly regulated process, and apoptosis appears to play an essential role in maintaining an appropriate number of germ cells that can be adequately supported and matured by the Sertoli cells [19]. Several authors have reported accelerated apoptosis of germ cells in infertile men with impaired spermatogenesis [9–12]. In the present study, the percentages of apoptotic germ cells were comparable to those reported in other studies. Only Tesarik et al. [9] reported much higher percentages of apoptotic germ cells in patients with incomplete spermatogenesis. The discrepancy between the studies might have been due to the method of apoptosis detection. Tesarik et al. examined the germ cell apoptosis by analyzing cell smears from mechanically disintegrated testicular tissues and used a FITC-labeled nucleotide to detect DNA fragmentation.

The mechanisms of the germ cell apoptotic process underling spermatogenesis impairment are poorly understood. In the current study, increased germ cell apoptosis was observed in patients with AZFc deletions in comparison to patients without AZFc deletions and patients with obstructive azoospermia. This increase in apoptosis may be responsible for the progressive loss in spermatogenic potential. Rajpurkar *et al.* [20] demonstrated that chronic cigarette smoke induced apoptosis in rat testis. They concluded that increased apoptosis might be one of the pathogenic mechanisms responsible for defective spermatogenesis in the rat following chronic cigarette smoking. A varicocele has a progressively toxic effect on the testes that may ultimately result in irreversible infertitity [21]. Hassan et al. [22] reported that the percentage of apoptotic cells in seminiferous tubules of infertile patients with varicocele was significantly higher than in patients with obstructive azoospermia (6.29% vs. 2.71%). These percentages of apoptotic germ cells were comparable to those reported herein.

AZFc contains five protein-coding gene families (BPY2, CDY, DAZ, CSPG4LY and GOLGA2LY), which are all transcribed in testicular tissue [23]. These genes are thought to be associated with spermatogenesis, but their function is unknown. The best-characterized gene family in the AZFc region is the DAZ gene. The DAZ gene family encodes a protein with an RNA-binding motif, suggesting a functional role in mRNA stability or in the translational regulation of its target RNA. The CDC25 family has been recognized as the downstream target of DAZL, which is the autosomal DAZ family gene [24, 25]. CDC25 phosphatases play a key role in cell cycle progression by controlling the activation of cyclin-dependent kinases [26]. Of the CDC25 family, CDC25A is expressed at a high level in the testis, suggesting that CDC25A plays a crucial role in the mitotic or meiotic regulation of spermatogenesis [27, 28]. Inactivation of CDC25 induces cell cycle arrest and apoptosis of hepatocellular carcinoma cells [29]. The inhibition of the CDC25 function, owing to a loss of DAZ genes, may contribute to the accelerated germ cell apoptosis observed in patients with AZFc deletions.

This is the first paper reporting increased apoptosis of germ cells in patients with AZFc deletions. Further studies with a larger population are needed to confirm these results.

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Ⅱ．感染症

7．風疹ウイルス

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風疹は風疹ウイルスによる感染症であるが，疾患そのものは「三日ばしか」ともよばれ，重篤な転帰をとることはまれな疾患である．しかしながら，妊婦が妊娠初期に風疹に罹患した場合，出生児に「先天性風疹症候群」が高率に発症することから，産婦人科領域では重要な疾患である．本稿においては，風疹感染，先天性風疹症候群の診断およびそれに関連した検査事項について解説する．

風疹の臨床経過および診断

風疹（rubella）は，風疹ウイルスにより発症する急性の発疹性疾患である．風疹ウイルスは，Togavirus 科 Rubivirus 属に属する直径 60～70 nm の一本鎖 RNA ウイルスで，血清学的には亜型のない単一のウイルスである．風疹の臨床経過を図 1 に示したが，潜伏期は 16～18 日で，小児では鼻汁，下痢などとともにリンパ節の腫脹（耳介後部，後頭部，頸部など）が認められる．これに引き続き発疹（小紅斑状で全身に出現する）が認められることが多い．成人では微熱の後，食欲不振，倦怠感，結膜炎，上気道炎様症状が認められ，小児と同様リンパ節腫脹が認められ，その後発疹が認められることが多い．上気道から分泌されたウイルスによる飛沫感染形式を取るが，感染力は麻疹，水痘などに比べ弱い[1)～3)]．

風疹の確定的な診断には他のウイルス疾患と同様にウイルスの分離が重要であるが，実地臨床ではほとんど行われていない．すなわち上述した発熱，発疹，リンパ節腫脹の 3 主徴が認められた場合風疹を疑い，他の発疹性疾患との鑑別のため，血清学的診断が行われる．血清学的診断として，赤血球凝集抑制反応法（hemagglutination inhibition：HI 法），中和法（neutralization test：NT 法），補体結合法（complement fixation：CF 法），酵素免疫測定法（enzyme linked immunosorbent assay：ELISA 法）などがあるが，通常用いられるものは HI 法による抗体測定および酵素免疫測定法によるグロブリン別風疹抗体すなわち，風疹 IgG 抗体，IgM 抗体測定である．風疹感染に伴うこれらの抗体の推移を図 1 に示した．

発疹出現後 48 時間以内に HI 抗体価が上昇し，その値は発疹発現後ほぼ 1 週間でピークに達する．これと同時期に風疹 IgM 抗体が上昇し 4～5 週間で低下するため，IgM 抗体が陽性である場合最近の風疹感染の可能性があり診断上有用とされてきた．しかしながら，風疹 IgM 抗体が長期にわたり陽性を示すこともあり，その判断には注意を要する．風疹 IgG 抗体は，IgM 抗体が低下した後上昇するため，IgG 抗体が陽性，IgM 抗体が陰性であれば最近の感染は否定的となる．

風疹の流行についてであるが，以前は 2～3 年の周期で流行し，10 年ごとに大流行がみら

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れていたが，ここ25年ほどの間では，1982年，1987年，1992年に大きい流行が認められたが，その発生数および流行の規模は次第に縮小しつつある．

一般的な風疹の診断は前述のようにしてなされ，通常は対症的な治療を行い，自然治癒を待つこととなる．これに対し，妊婦に関して風疹感染が疑われた場合，後述の「先天性風疹症候群」発症との関係で，感染の有無の判断およびその対応が極めて重要となる[4)～6)]．次項においてはその点について述べる．

妊婦における風疹感染とその対応

現在のわが国の妊婦健診においては，ほぼ全例に風疹抗体検査（HI法による）が実施されている状況であり，先天性風疹症候群の発症率は極めて低いものである．しかしながら，2004年，それまで年間1例程度であった先天性風疹症候群の発症が半年で5例報告されたことにより，厚生労働省研究班が「風疹流行および先天性風疹症候群の発生抑制に関する緊急提言」を行った[4)5)]．

同提言では，妊娠初期検査の一環として施行されている風疹抗体検査とともに，問診の重要性を指摘しており，「妊娠中の発疹の有無」「風疹患者との濃厚な接触の有無」について聴取することを勧めている．

問診で該当項目がない場合，風疹抗体価により表1のような対応を行う．HI抗体価が陰性あるいは16倍以下の場合には風疹ウイルスに対する免疫がないかあるいは不十分と判断し，風疹患者のいる場所，人込みを避けさせること，同居家族への風疹ワクチン接種の推奨などの指導を行う．HI抗体価が32～128倍の場合適度

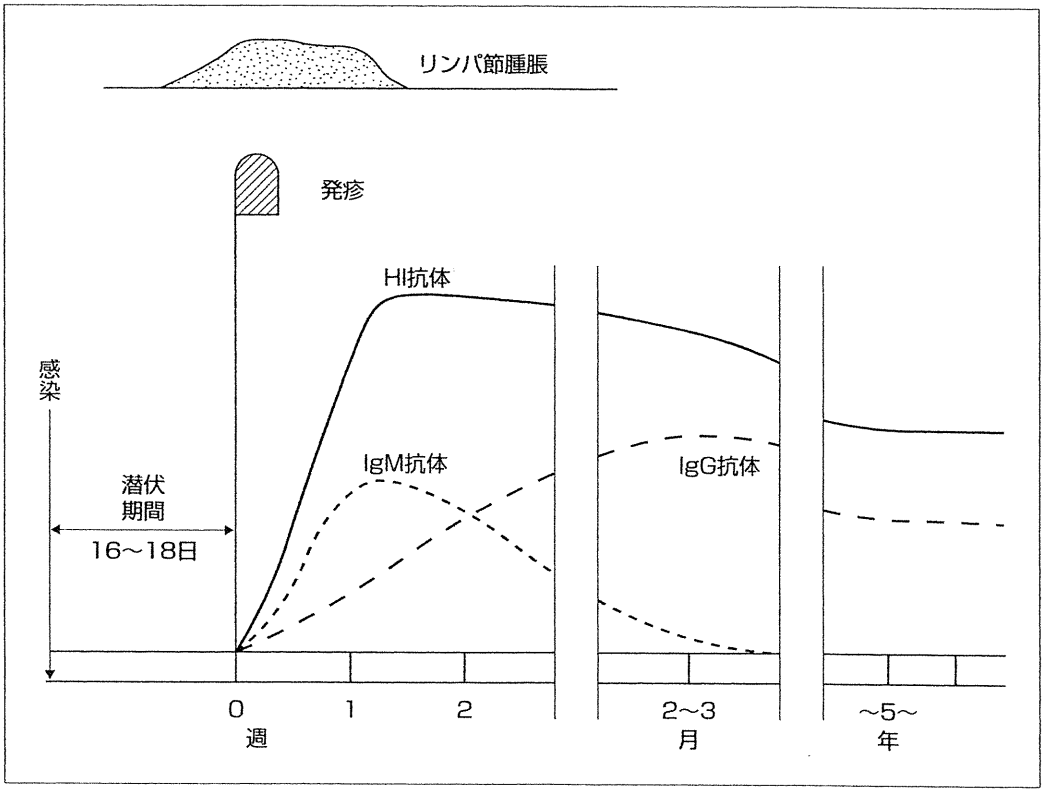

図1　風疹感染の経過と抗体の推移

＊HI抗体：ピーク時は256倍～2,048倍，時間の経過とともに32倍から128倍で安定
（ただし，時間が経過してからの抗体価は幅がある）

表１　妊娠初期検査における風疹抗体価による対応
（問診により風疹感染を考慮させる状況にない場合）

風疹抗体価	取り扱い
陰性または 16 倍以下	妊娠中の注意指導 　人込みや子どもの多い場所を避ける 　風疹患者のいる場所を避ける 夫、子供および同居家族へのワクチン接種推奨 分娩後早期のワクチン接種の推奨
32 ～ 128 倍	以後の妊娠の経過中、以下に該当するか 　妊娠中の発疹 　風疹患者との濃厚な接触 なければ終了 あれば「256 倍以上」の取り扱いへ
256 倍以上	再度血清 HI および風疹 IgM を測定 　HI 不変かつ IgM 陰性：終了 　HI が 4 倍以上の上昇または IgM 陽性 　　各地区相談窓口（2 次施設）へ相談

な免疫を保有していると判断し，256 倍以上では最近の風疹感染の可能性も考慮し，対応する．ただし，256 倍以上であるとしても風疹感染である可能性は極めて低く[4]，妊婦に過剰な不安を抱かせないことが重要である．

問診で該当項目がある場合には，ペア血清で風疹 HI 抗体および風疹 IgM 抗体を測定する．HI 抗体が陰性あるいは 16 倍以下の場合には表 1 の項目に従う．ペア血清検査で HI 不変かつ IgM 陰性の場合には終了とし，HI が 4 倍以上の上昇を示すか IgM が陽性の場合には，文献 5 に示されている各地区ブロックごとの相談窓口（二次施設）へ相談することが勧められている．これに対し，風疹 IgM 抗体が弱陽性の場合など実際の臨床の場で対応に苦慮することもまれではない．すなわち，多くの医療施設にとって遠隔地である二次施設へ紹介すべきか否か迷うことも多い．

このことに関連して，日本産科婦人科学会編集の「産婦人科診療ガイドライン 2008」では「Persistent IgM」という概念を指摘している．現時点で完全にコンセンサスを得られたものではないが，その基準として，①低レベル，②1 ～ 2 カ月後の再検でもほぼ同じ値で検出，③高い IgG 抗体価，を提示している[7]．また，奥田らは，風疹 IgM 抗体のカットオフ値は基本的には感染後 2 ～ 3 カ月で陰性となるよう設定されているものの，低レベルの陽性が 3 年以上持続する例も存在することから，IgM が陽性であっても直ちに最近の感染とはいえないと指摘している[4]．したがって，上記の「Persistent IgM」の条件を満たす症例に対しては，状況を説明のうえ，希望があればそのまま経過を観察することも選択肢の一つと判断される．筆者らはそのような症例を経験しており，その経緯について紹介している[8]．ただし，風疹感染の可能性が否定しきれない場合には，二次施設への紹介を考慮することが重要である．

妊娠と風疹感染の最大の問題は，先天性風疹症候群であり，次項において解説する．

表2　先天性風疹症候群報告のための基準
（改正感染症法，2003年11月）

診断した医師の判断により，症状や所見から当該疾患が疑われ，かつ，以下の1）と2）の基準を両方とも満たすもの

1）臨床症状による基準
「Aから2項目以上」または「Aから1つと，Bから2つ以上」もしくは「Aの（2）または（3）と，B（1）」
A　（1）先天性白内障，または緑内症
　（2）先天性心疾患（動脈管開存，肺動脈狭窄，心室中隔欠損，心房中隔欠損など）
　（3）感音性難聴
B　（1）網膜症
　（2）骨端発育障害（X線診断によるもの）
　（3）低出生時体重
　（4）血小板減少性紫斑病（新生児期のもの）
　（5）肝脾腫

2）病原体診断等による基準
以下のいずれかの一つを満たし，出生後の風疹感染を除外できるもの
1．風疹ウイルスの分離陽性，またはウイルス遺伝子の検出
　　例：RT-PCR法など
2．血清中に風疹特異的IgM抗体の存在
3．血清中の風疹HI価が移行抗体の推移から予想される値を高く超えて持続
（出生児の風疹HI価が，月あたり1/2の低下率で低下していない）

先天性風疹症候群

症状および感染時期と胎児異常

妊娠初期に風疹に罹患した場合，児の先天性風疹症候群（congenital rubella syndrome：CRS）発症のリスクが高まる．発症率については各種の報告があるが，妊娠第1カ月の感染で約60%，第2カ月で80%，第3カ月で約50%，第4カ月で約20%，第5カ月では約15%などという報告がなされている[2]．CRSの三大症状は先天性心疾患，難聴，白内障である．このうち，白内障は妊娠3カ月以内，先天性心疾患は4カ月以内の風疹感染で発症することが知られているが，難聴は4カ月以後でも発症する可能性が指摘されている[2]．三大症状以外に網膜症，肝脾腫，血小板減少，糖尿病，発育遅滞，精神発達遅滞，小眼球症など多様な症状の発現が報告されている．

先天性風疹症候群の診断

出生した児に先天性心疾患，難聴，白内障などのCRSを疑わせる症状が認められる場合には，ウイルス学的検査あるいは免疫学的検査により診断を行う．表2にその診断基準を示した[3]．

臨床症状としてA項目，B項目があるが，A項目には，①先天性白内障もしくは緑内障，②先天性心疾患，③感音性難聴が含まれ，B項目として，①網膜症，②骨端発育障害，③低出生体重児，④血小板減少性紫斑病，⑤肝脾腫が含まれる．また検査項目（病原体診断など）の基準として，①風疹ウイルスの分離陽性，またはウイルス遺伝子の検出，②血清中に風疹特異的IgM抗体の存在，③血清中の風疹HI価が移行抗体の推移から予想される値を高く超えて持続，

が含まれる．これらの臨床症状による基準，臨床検査による基準の組み合わせにより，CRSの診断がなされる．

CRSは感染症新法により全例報告の義務があり，診断した医師は7日以内に最寄りの保健所に届け出る必要がある．

先天性風疹症候群の予防

予防で重要なことは，妊娠可能年齢の女性が十分高い風疹抗体価を保有することである．わが国における風疹ワクチンの定期接種については，1977年から女子中学生を対象とした接種が開始されたが，1994年の予防接種法改正により，対象が生後12カ月以上～90カ月未満の男女（標準は生後12カ月以上～36カ月以下）とされた（図2）[3]．

その後さらに改正され2006年からは，生後12～24カ月（第1期）および就学前1年間（第2期）の男女となっている．ただし平成15年の推計では20歳台，30歳台の女性において約70万人が風疹抗体陰性であることが指摘されている[4]．自然感染によりすでに明らかに風疹ウイルスに対する免疫が成立している場合以外は，風疹ワクチンによる能動免疫により風疹抗体を陽転させておくことが重要である．

ただし，風疹ワクチンは弱毒生ワクチンであり，妊娠中のワクチン接種は避けることが必要である．風疹ワクチンに関する薬剤説明（乾燥弱毒生風疹ワクチン「タケダ」を例示）では，接種不適当者として「妊娠していることが明らかな者」が挙げられており，また基本的注意事項として「本剤は妊娠可能な婦人においては，あらかじめ約1カ月間避妊した後接種すること，およびワクチン接種後約2カ月間は妊娠しないように注意させること」との記載があり，注意を喚起している[9]．

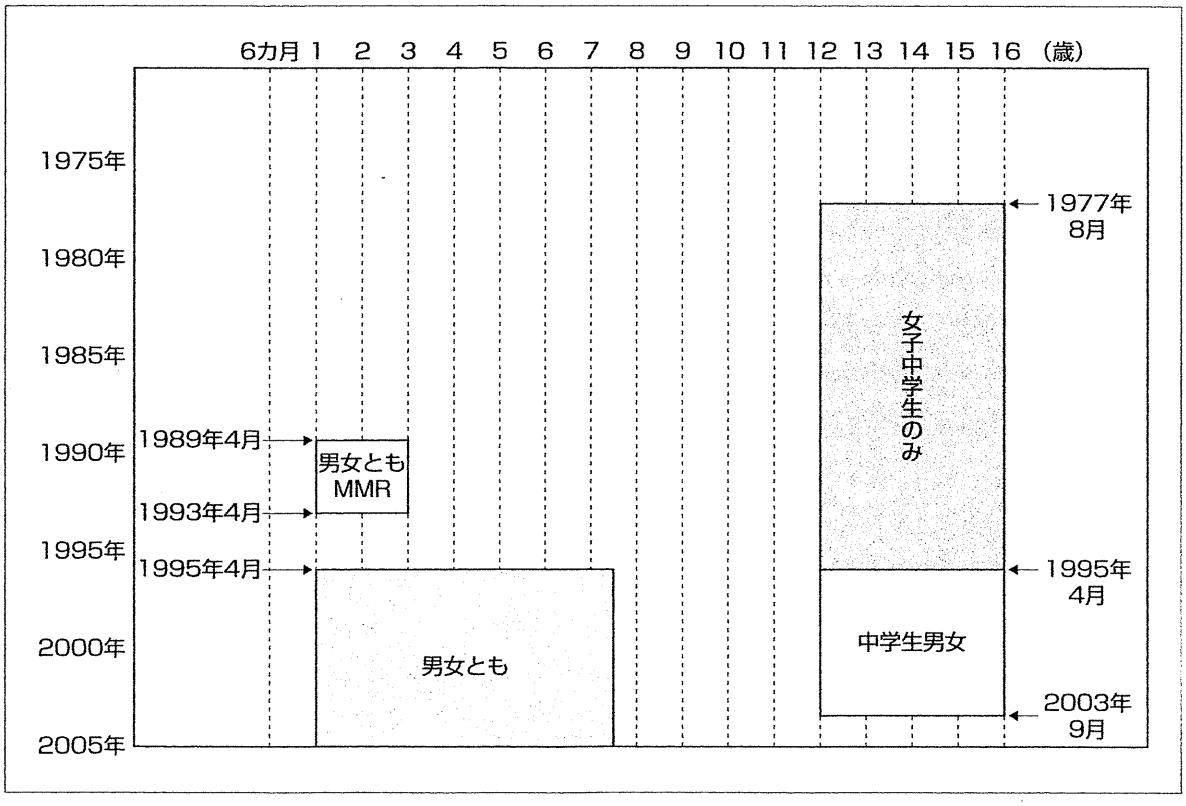

図2　わが国における風疹ワクチン定期接種の経緯（2005年まで）

［文献3）より引用］

風疹罹患に関する胎内診断

妊娠初期の風疹感染の可能性が否定しきれない場合には，上述のように二次施設への相談，紹介などを行うこととなり，さらに直接的な検査が行われることもある．胎児感染の有無を直接確認する方法として，胎児血，羊水，胎盤絨毛など胎児由来組織を用い風疹ウイルスRNAをRT-PCR（reverse transcriptase-polymerase chain reaction）法で増幅して検出する方法が応用されている．いずれも観血的検査であり，その実施に際しては十分な説明と同意が必要である．種村は，羊水では検出率がやや低く偽陰性例が存在すること，より正確な結果を得るには絨毛あるいは胎児血を用いることが望ましいこと，およびRT-PCRで陽性であってもCRSが発症するとは限らず，十分なカウンセリングが必要であることなどを指摘している[10]．

おわりに

妊婦が風疹に罹患した場合先天性風疹症候群発症のリスクがあり，産婦人科領域では重要な感染症である．診断に関しては風疹ウイルスに対する抗体検査が重要であり，検査値についてのより正確な判断が求められている．近年，先天性風疹症候群の発症頻度は極めて低いものとなっているが，産婦人科医としては，妊娠可能年齢にある女性に対し妊娠成立前の風疹抗体獲得の重要性をアピールし，先天性風疹症候群が根絶されるよう努力することが重要である．

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Article

Identification of Receptor Tyrosine Kinase, Discoidin Domain Receptor 1 (DDR1), as a Potential Biomarker for Serous Ovarian Cancer

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Abstract: Ovarian cancer, one of the most common gynecological malignancies, has an aggressive phenotype. It is necessary to develop novel and more effective treatment strategies against advanced disease. Protein tyrosine kinases (PTKs) play an important role in the signal transduction pathways involved in tumorigenesis, and represent potential targets for anticancer therapies. In this study, we performed cDNA subtraction following polymerase chain reaction (PCR) using degenerate oligonucleotide primers to identify specifically overexpressed PTKs in ovarian cancer. Three PTKs, janus kinase 1, insulin-like growth factor 1 receptor, and discoidin domain receptor 1 (DDR1), were identified and only DDR1 was overexpressed in all ovarian cancer tissues examined for the validation by quantitative real-time PCR. The DDR1 protein was expressed in 63% (42/67) of serous ovarian cancer tissue, whereas it was undetectable in normal ovarian surface epithelium. DDR1 was expressed significantly more frequently in high-grade (79%) and advanced stage (77%) tumors compared to low-grade (50%) and early stage (43%) tumors. The expression of the DDR1 protein significantly correlated with poor disease-free survival. Although its functional role and clinical utility remain to be examined in future studies, our results suggest that the expression of DDR1 may serve as both a potential biomarker and a molecular target for advanced ovarian cancer.

Keywords: ovarian cancer; tyrosine kinase; DDR1; disease biomarker; cDNA subtraction; degenerate PCR

1. Introduction

Ovarian cancer, one of the most common gynecological malignancies, is an aggressive cancer associated with high morbidity and mortality, especially in the case of advanced disease. Unfortunately, ovarian cancer is rarely diagnosed in its early, most curable stages, and the tumors are already disseminated abdominally in 75% of patients at the time of diagnosis. The tumor grade, histological type, and presence of residual disease after initial surgery are also important clinicopathological factors related to patient outcome [1]. To improve the prognosis of ovarian cancer, it is vital to clarify the molecular mechanisms involved in the progression of this disease, and to develop novel and more effective treatment strategies against advanced disease.

Protein tyrosine kinases (PTKs) play an important role in the signal transduction pathways that control cell proliferation and differentiation, and are involved in tumorigenesis. Many PTKs have been shown to act as oncogenes, and analysis of PTK expression in malignant cells will lead to a better understanding of oncogenesis, which in turn may lead to novel therapies based on selective inhibition of the PTKs involved in malignant transformation [2]. Various targeted therapeutics have been explored for the management of ovarian cancer. PTKs such as Her2/neu, the epidermal growth factor receptor (EGFR), and vascular endothelial growth factor (VEGF), represent potential targets for ovarian cancer, and agents targeting these molecules are already being used in the clinic for other diseases [3–5]. The PTKs may also provide an important predictive marker for therapeutic response and patient outcome [6,7]. It is important to identify predictive PTKs to identify targeted subpopulations of patients who will respond to both the existing tyrosine kinase inhibitors (TKIs) and to new agents developed against new targets, and to obtain a better understanding of the underlying mechanisms of resistance to the existing agents so that new compounds can be developed to overcome this resistance.

The expression of PTKs can easily be determined by RT-PCR using degenerate primers which recognize common, relatively invariable cDNA sequences of members of the PTK family. PCR-based cDNA subtraction offers an efficient method for selectively amplifying differentially expressed genes. This method is particularly well-suited for the identification of target cDNAs that correspond to rare transcripts, which are typically the most difficult to obtain.

In this study, we have combined cDNA subtraction and polymerase chain reaction (PCR) using degenerate oligonucleotide primers representing conserved amino acid sequences within the tyrosine kinase domain to identify specifically overexpressed PTKs in serous ovarian cancer. The PTKs that were identified were subsequently examined for their potential association with the clinicopathological factors and patient outcome.

2. Materials and Methods

2.1. cDNA Subtraction and Degenerate PCR

Primary serous papillary ovarian cancer tissue samples and their corresponding normal ovarian tissue samples were obtained from three patients who underwent primary debulking surgery at the Niigata University Medical and Dental Hospital. Total RNA was extracted using an RNeasy Miniprep Kit (Qiagen), and poly(A)RNA was isolated using the FastTrack 2.0 Kit (Invitrogen) according to the manufacturer's instructions. The cDNA was synthesized, and cDNA subtraction was performed for each patient using a PCR-select cDNA subtraction kit (Clontech) according to the manufacturer's instructions. To select truly overexpressed PTKs in the cancer tissue, cDNA subtraction was performed between the tumor cDNA as a tester and 5-times the amount of normal ovarian cDNA as a driver. The subtracted cDNA fragments were amplified by suppression PCR in order to enrich the differentially expressed sequence and to reduce background. The final products of cDNA subtraction were further amplified with primers corresponding to consensus sequences for PTKs. Primers were synthesized corresponding to the amino acids HRDLAARN and DVWS(F/Y)G(I/V), which are highly conserved sequences in the catalytic domains of PTKs. The products of the degenerate PCR were subcloned and then sequenced.

2.2. Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction Assay

Quantitative RT-PCR was performed to validate the expression of each clone identified by cDNA subtraction and degenerate PCR using Taqman probes. Tumor samples from 8 patients with serous ovarian cancer and their corresponding normal ovarian tissue were used for the analysis. Total RNA was isolated and processed with the RNeasy Miniprep Kit (Qiagen). A TaqMan two-step kit (Applied Biosystems) was utilized for all RT reactions. The High Capacity cDNA Archive kit utilizes a proprietary mixture of 10 × random primers, MultiScribe ™ Reverse Transcriptase (50 U/μL) and 10 × Reverse Transcription Buffer and 25 × dNTPs for the RT reaction. RT reactions were incubated at 25 °C for 10 min, followed by 37 °C for 120 min in a thermal cycler. After cDNA synthesis was performed during the second step, RT-PCR was then performed in an ABI PRISM 7900 in a 50 μL final volume with 1 μL of the cDNA template, 10 μM of primers and 5 μM of TaqMan Probe, and enzymes from the 2 × TaqMan Universal PCR Master Mix (ABI) according to the manufacturer's protocol. The thermal cycling conditions were: 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The primer sequences were designed by Operon Biotechnology (Tokyo, Japan), and were as follows; IGF1-R: forward primer: 5'-ACTTCTGCGCCAACATCCTCA-3′; reverse primer: 5'-CCCTTTAGTCCCCGTCACTTCC-3', JAK1: forward primer: 5'-AGAGGCATATAAAATTTAGATTGC-3'; reverse primer: 5′-TGTCCTTGTTGAGAGTGAACA-3', DDR1: forward primer: 5'-ATGGAGCAACCACAGCTTCTC-3'; reverse primer: 5'-CTCAGCCGGTCAAACTCAAACT-3', and GAPDH: forward primer: 5'-GGCTCCCACCTTTCTCATCC-3'; reverse primer: 5'-GATGTGGGGAGTACGCTGC-3'. The TaqMan-probes were obtained from Applied Biosystems.

2.3. Immunohistochemistry

Archival tissue from patients with ovarian cancer removed at debulking surgery and normal ovaries removed during surgery for benign conditions were used for immunohistochemical analysis. H&E-stained sections of each sample were reviewed by a pathologist, and areas corresponding to tumor tissue were marked. Immunohistochemistry (IHC) was performed on 67 serous ovarian cancer tissue specimens and 5 normal ovarian tissue specimens that were paraffin-embedded and cut into 4-μm-thick sections and mounted on positive charge-coated slides. Tissue sections were dried overnight in a 45 °C oven. For antigen visualization, the EnVision/HRP system and DAB+ (Dako) were used. The immunohistochemical procedure was optimized by testing different antigen retrieval methods using negative and positive controls. An anti-DDR1 antibody (Santacruz) was added at a 1:100 dilution to each section and incubated for 60 min at room temperature. The IHC results were scored based on the staining intensity as negative and positive. Immunoreactivity was scored as follows: the numbers of DDR1 positive cells were counted out of 100 cells in three different high power fields and judged as "positive" when >50% of cells were positively stained according to previous reports [8]. Cases were classified into two groups: group 1 (negative) included the cases with negative staining and less than 50% of staining, group 2 (positive) included cases with more than 50% of staining.

2.4. Association between the Expression of DDR1 Protein in Ovarian Cancer and the Clinical Disease Stage, Tumor Grade, and Patient Outcome

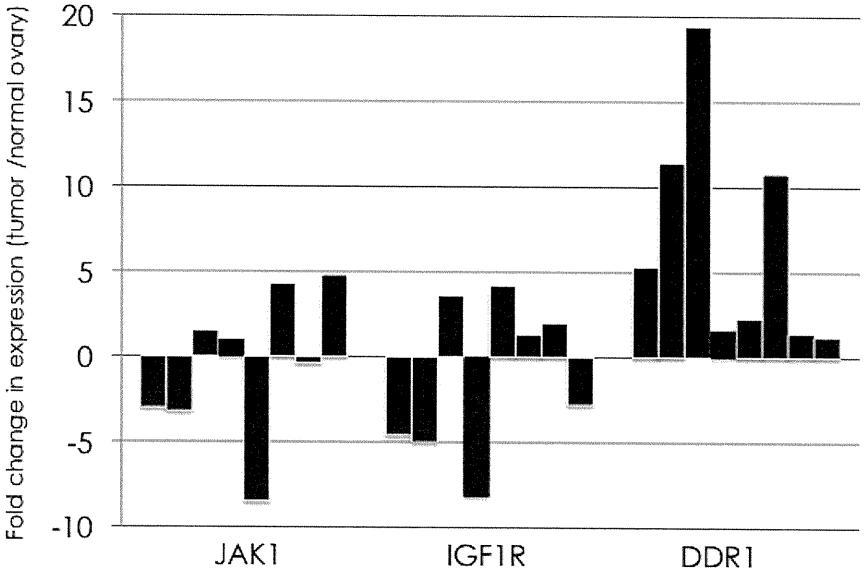
The clinical data for this immunohistochemical staining study were collected from 67 consecutive unselected patients with primary epithelial ovarian cancer who had undergone surgery at the Niigata University Dental and Medical Hospital between 2000 and 2004. Only patients with a histological diagnosis of serous papillary ovarian cancer were included in this study, and those with borderline tumors were excluded. All tumors were graded and staged according to the FIGO (International Federation of Gynecology and Obstetrics) classification. All patients provided consent according to the institutional review board of Niigata University Dental and Medical Hospital, Niigata, Japan.

3. Results

After screening ovarian cancer tissue samples by the PCR-based cDNA subtraction and degenerate PCR for selection of the clones of differentially overexpressed PTKs, we randomly selected 202 clones. By sequencing analyses, 140 clones were revealed to be identical to janus kinase 1 (JAK1), 30 to the insulin-like growth factor 1 receptor (IGF1-R), and eight to the discoidin domain receptor 1 (DDR1). The other 24 clones were unidentified genes.

We performed quantitative real-time PCR to validate whether the three PTKs were constitutively overexpressed in ovarian cancer using eight serous ovarian cancer tissue and their corresponding normal ovarian tissue samples. The DDR1 gene was overexpressed in all eight individuals, whereas the expression levels of JAK1 and IGF1-R were not increased in half of the ovarian cancer tissue samples (Figure 1). The DDR1 expression level in the ovarian cancer tissue samples was increased 6.7 fold on average compared with normal ovarian tissue.

Figure 1. Quantitative real-time PCR for the validation of overexpression of three identified genes in ovarian cancer. Quantitative real-time PCR was performed to validate the overexpression of three identified PTKs, JAK1, IGF1R, and DDR1, in ovarian cancer. Fold change in the expression between ovarian cancer tissue and their corresponding normal ovarian tissue samples are shown with black bars. The DDR1 gene was overexpressed in all eight individuals, whereas the expression levels of JAK1 and IGF1-R were not increased in half of the ovarian cancer tissue samples.



To characterize the expression of DDR1, we carried out immunohistochemical analysis of 67 primary serous ovarian cancers from patients who had not received any prior treatment, obtained from primary debulking surgery. As demonstrated in Figure 2, no DDR1 staining was present in epithelial cells in normal ovary and serous adenoma, but the expression was increased in the ovarian cancer cells.

The DDR1 protein was highly expressed in 69% (46/67) of serous ovarian cancer tissue samples. Moreover, DDR1 protein expression was correlated with the pathologic grade of the tumor and the clinical disease stage at the time of surgery. As shown in Table 1, DDR1 was expressed significantly more frequently in FIGO Grade 1 (50%) compared to combined G2 and G3 (79%) tumors ($p = 0.015$).

We next compared the clinical disease stage for patients with different levels of DDR1 expression. Given the limited number of samples, the stage I and II samples were combined (early stage), as were the stage III and IV samples (advanced stage). The expression of DDR1 in early stage samples was 50%, while it was 82% in advanced stage tumors. There were significant differences in DDR1 expression found between advanced stage tumors compared with tumors found in the early stage ($p = 0.006$) (Table 2).

DDR1 expression was then examined for an association with disease-free survival and overall survival using Kaplan-Meier survival analysis with the log-rank statistic to determine significance. Kaplan-Meier survival curves generated for tumor DDR1, high *versus* low expression, are given in Figure 3. High tumor DDR1 expression was significantly associated with a poor outcome for disease-free survival ($p = 0.032$). With regard to overall survival, high tumor DDR1 expression showed a tendency toward a poorer outcome, but this trend was not statistically significant ($p = 0.064$).

Figure 2. Representative immunohistochemistry staining of DDR1. Immunohistochemical analysis with the DDR1 antibody revealed negative DDR1 protein expression in (A) normal ovary and (B) serous cystadenoma, whereas positive expression was observed in (C) serous ovarian cancer cells. (A) no DDR1 staining in surface epithelium of normal ovary (black arrow); (B) no DDR1 staining in epithelial lining cells in serous cystadenoma (black arrow); (C) representative staining pattern of serous ovarian cancer tissue shows positive DDR1 staining in most serous adenocarcinoma cells (black arrow) with negative DDR1 staining in stromal cells (white arrow); (D) in placenta (positive control); all trophoblastic cells show positive DDR1 staining. Original magnification: ×40, scale bar: 50 μm (white bar).

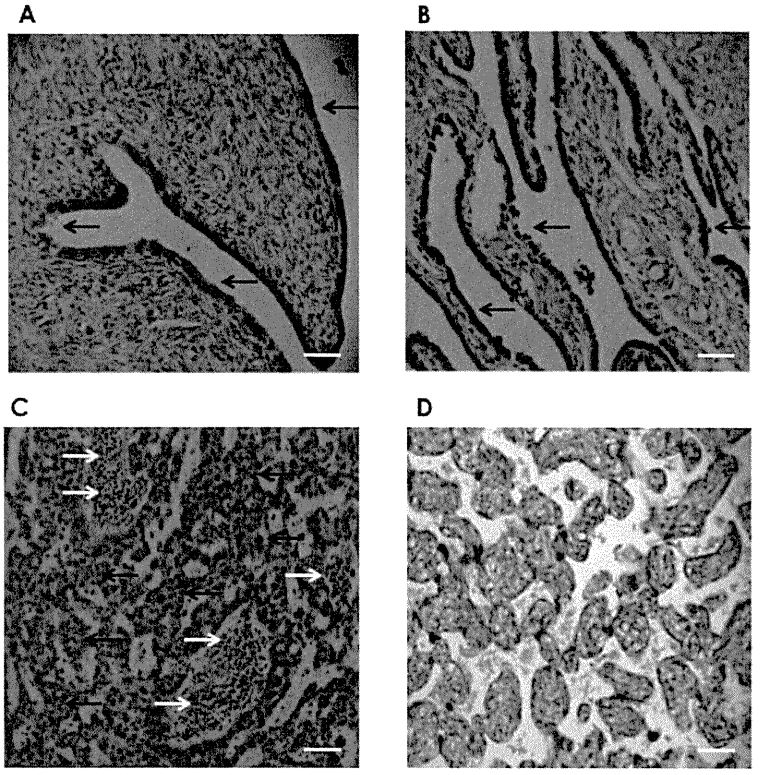


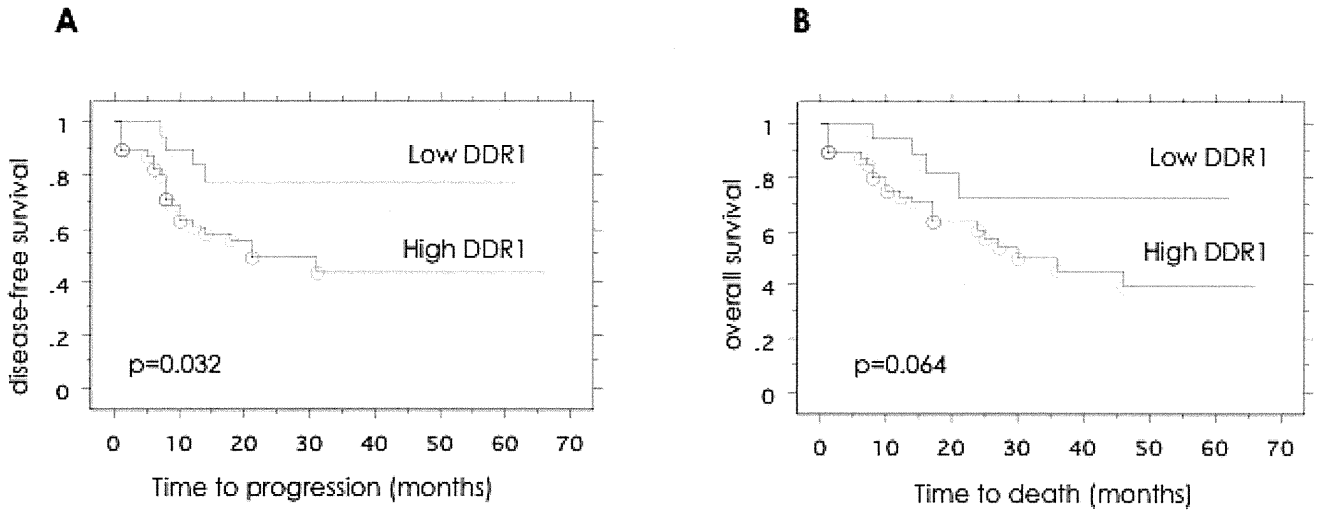
Table 1. DDR1 expression in patients with ovarian cancer according to tumor grading.

	DDR1 positive	DDR1 negative	
G1	12	12	
G2	21	5	$P = 0.015$
G3	13	4	

Table 2. DDR1 expression in patients with ovarian cancer according to clinical staging.

Stage	Cases No.	DDR1 positive	Percent of DDR1 positive	
I	22	11	50.0	$p = 0.006$
II	6	3		
III	35	29	82.1	
IV	4	3		
total	67	46	68.7	

Figure 3. Patient outcome according to the DDR1 expression in patients with serous ovarian cancer. Kaplan-Meier analysis of (A) disease-free survival and (B) overall survival, according to DDR1 expression levels. Significant trend for shorter disease-free survival was observed in the DDR1 positive group ($p = 0.043$).



4. Discussion

In the present study, we identified DDR1 as a differentially expressed PTK gene in primary epithelial serous ovarian cancer using a combination of cDNA subtraction and degenerate PCR-based cloning. DDR1 was more highly expressed in ovarian cancer samples compared with normal ovarian tissues. We were able to show that DDR1 expression is associated with the tumor grade and clinical disease stage, and is inversely correlated with the survival outcome of patients.

Receptor tyrosine kinases control a wide array of cellular responses, including the regulation of cell growth, differentiation, migration, metabolism, and survival. DDR1 was independently isolated as a novel receptor tyrosine kinase by several laboratories from human, mouse, and rat tissue in the 1990s [9–11]. DDR1 is characterized by a structural domain of 160 amino acids in its extracellular part that exhibits strong sequence similarity to the *Dictyostelium discoideum* protein discoidin 1, coagulation factors V and VIII, and to a *Xenopus laevis* recognition protein, A5. DDR1 is activated by collagen type I, II, III, V, and XI. Activation of DDR1 by collagen results in its sustained intracellular phosphorylation. DDR1 is widely expressed in epithelial cells of both fetal and adult organs. Although the physiological

functions of DDR1 are not fully understood, DDR1 signaling is essential for cerebellar granule differentiation [12], arterial wound repair [13], and mammary gland development [14]. It is clear that DDR1 is involved in cell interactions with the extracellular matrix, and that it controls adhesion and cell motility [15,16].

DDR1 was found to be overexpressed in breast, brain, colon, and lung cancers, thus suggesting that this receptor may play a role in the tumorigenesis of epithelial cancers [17–20]. In breast cancer, DDR1 was overexpressed in both primary breast tumor samples and metastasis-containing lymph nodes [21]. DDR1 protein levels were elevated in 100% of patients with primary and metastatic brain tumors [18], in 61% of patients with non-small cell lung cancer, and in 64% of patients with invasive lung adenocarcinoma [22]. Thus, DDR1 expression appears to be elevated in a variety of human cancers. Consistent with studies of DDR1 in these solid tumors, elevated levels of DDR1 were seen in serous ovarian cancer in this study. Our results were also consistent with a study by Heinzelmann-Schwarz *et al.* that reported that DDR1 proteins are highly overexpressed in all histological subtypes of epithelial ovarian cancer compared with the normal ovarian surface epithelium [23].

The overexpression of DDR1 in these different human cancers suggests that it may have a function in tumor progression. It has been reported that DDR1 is overexpressed in high-grade brain, esophageal, and breast cancers, and high expression of DDR1 was associated with a significantly poorer survival in several cohorts of patients with brain, breast, and lung cancers [18,22,24–26]. In the present study, we showed that DDR1 expression was associated with high-grade and advanced stage tumors, as well as with poor survival in patients with ovarian cancer. These results were not consistent with those from previous study by Heinzelmann-Schwarz *et al.* [23]. They reported that expression of membranous DDR1 did not correlate with survival of patients. Recently Mihai C. *et al.* proposed the model of the DDR1 activation mechanism by analyzing the cellular distribution of DDR1 [27]. They showed the aggregation and cellular internalization of the receptor following collagen stimulation. In this study, we evaluated both membranous and cytoplasmic DDR1 expression and did not analyze the intensity of the staining. Although the activation process of DDR1 still remains largely unknown, evaluation of the cellular distribution or intensity of DDR1 expression might be required to analyze the precise biological activity of DDR1. Several recent studies have examined the molecular mechanisms underlying the role of DDR1 in tumor progression, invasion, and metastasis. One study showed that DDR1 expression may be regulated during the cell cycle, because overexpression of p53 in osteosarcoma cells induces DDR1 expression [28]. Ongusaha *et al.* have reported that DDR1 is a direct p53 transcriptional target, and that inhibition of DDR1 function resulted in increased apoptosis through a caspase-dependent pathway [29]. Other published data imply that the Wnt-5a pathway may overlap with DDR1 signaling [26]. Shintani *et al.* have shown that DDR1 promotes the epithelial to mesenchymal transition in response to collagen I stimulation in human pancreatic cancer cells [30]. Although experimental evidence argues against a classification of DDR1 as a transforming oncogene, subsequent steps after the initial cellular transformation, such as invasion and metastasis, might be mediated by DDR1.

One of the major characteristics of an ideal biomarker or target for molecular therapeutics is that it is absent in benign tissue and present in the targeted malignancies. Our results showed no protein expression of DDR1 in normal ovarian epithelial cells. It has been reported that DDR1 expression is highest in the brain, lungs, placenta and kidneys, and is present at low levels in various other adult

tissues, such as melanocytes, the heart, liver, skeletal muscle, pancreas, and ovaries [31]. Elevated levels of DDR1 protein expression appear to be highly predictive of the presence of ovarian cancer. Although it would be nonspecific for ovarian cancer (because it could also indicate various other malignancies), the levels of DDR1 expression in body fluid or serum may have clinical prognostic utility as a biomarker for cancer.

Various targeted therapeutics have been explored for the management of ovarian cancer. In addition, numerous studies have examined the use of TKIs, including monoclonal antibodies against Her2/neu [4], other EGFRs [3], and VEGF [5], and small molecule tyrosine kinase inhibitors targeting various other receptors, including the EGFR and VEGFR [32,33]. A recent study showed that dasatinib, a multi-targeted TKI, inhibits DDR1, in addition to inhibiting BCR-ABL [34]. Moreover, DDR1 has also been identified as a potential target of other BCR-ABL inhibitors, including imatinib [35]. These inhibitors of DDR1 may prove to be therapeutically beneficial for the treatment of advanced ovarian cancer.

5. Conclusion

In summary, we have identified DDR1 as a differentially overexpressed PTK in ovarian cancer tissue using a combination of cDNA subtraction and degenerate PCR-based cloning. The association between DDR1 expression, tumor grade, clinical disease stage, and patient outcome suggests an *in vivo* role for this signal transduction pathway in ovarian cancer. The mechanisms by which DDR1 affects signaling cascades involved in tumor progression, invasion, and metastasis have not yet been fully characterized. Further investigation of DDR1 as a clinical biomarker and as a therapeutic target is warranted, especially for ovarian cancer.

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