表. 実験室診断で風疹の感染が確認された症例

症例	発症日	採取日	臨床検体	性別	年齢	風疹ワクチン歴	風疹IgM	風疹PCR			風疹ウイルス遺伝子型	症状	接触歴
								血液	咽頭ぬぐい	尿			
1	2011/2/10	2011/2/17	血液・咽頭ぬぐい・尿	男	40y	無記載	9.63	−	−	−		発熱(39.7℃)・発疹	勤務先での集団発生
2	2011/2/11	2011/2/18	血液・咽頭ぬぐい・尿	男	30y	無記載	10.35	−	−	−		倦怠感、発疹、目の充血	勤務先での集団発生
3	2011/2/14	2011/2/14 2011/2/16	血清 血清	男	35y	不明	− 1.39	+ −			2B	発疹	勤務先での集団発生
4	2011/3/3	2011/3/4	血液・咽頭ぬぐい	女	19y	無記載	N.D.**	−	+			発熱(37.5℃)・発疹・リンパ節腫脹(後頸部)・結膜炎	
5	2011/3/16	2011/3/30	血液・咽頭ぬぐい・尿	男	43y	不明	2.03	+	+	+		発熱(37.2℃)・発疹・リンパ節腫脹・上気道炎・結膜充血・筋肉痛	接触歴なし
6	2011/3/18	2011/3/19	血液・咽頭ぬぐい・尿	男	39y	無記載	N.D.**	−	+	−		発熱(37.3℃)・発疹(紅斑)	
7	2011/3/26	2011/4/1	血液・咽頭ぬぐい・尿	男	21y	無記載	9.7	−	−	−		発熱・咳・結膜充血・発疹・リンパ節腫脹	不明
8*	2011/3/31	2011/4/2	血液・咽頭ぬぐい・尿	男	22y	無記載	N.D.**	−	+	−		発熱・発疹・リンパ節腫脹(頸部)・結膜炎	渡航歴あり (上海3/14〜3/18)
9	2011/4/10	2011/4/15	血液・咽頭ぬぐい・尿	男	24y	無	8.74	−	−	+		発熱(38℃)・発疹・コプリック斑	接触歴なし
10	2011/4/19	2011/4/21	血液・咽頭ぬぐい・尿	男	47y	不明	8.08	−	+	+		発熱(37℃)・発疹・結膜充血	接触歴なし
11	2011/4/26	2011/5/2	血液・咽頭ぬぐい・尿	男	28y	不明	3.87	+	+	+		咽頭痛・鼻汁・全身性の発疹(発熱、結膜充血などはなし)	勤務先で麻疹患者あり
12	2011/4/28	2011/5/7	血清・咽頭ぬぐい・尿	男	39y	無記載	7.75	−	−	−		発熱(38℃)・発疹・コプリック斑	接触歴なし
13	2011/4/29	2011/4/30	血液・咽頭ぬぐい・尿	男	44y	無記載	−	+	+	−		発熱(38.2℃)・発疹・関節痛・筋肉痛・上気道炎	接触歴なし
14	2011/5/1	2011/5/2	血液・咽頭ぬぐい・尿	男	39y	不明	4.42	−	+	+		発熱・発疹・頭痛・関節痛	勤務先で流行 (2ヶ月間で麻疹疑い14〜5人)
15	2011/5/3	2011/5/6	血清	男	38y	無記載	2.72	−				発熱(37.9℃)・発疹・コプリック斑・角結膜炎	接触歴無し
16	2011/5/3	2011/5/9	血液・咽頭ぬぐい・尿	男	32y	無記載	7.7	−	−	−		発熱・発疹・咳嗽・鼻汁	千葉県船橋市に在住 (東京都内で仕事)
17	2011/5/8	2011/5/9	血液・咽頭ぬぐい	男	34y	不明	1.9	+	+		2B	発熱・発疹・コプリック斑・結膜炎	接触歴なし
18	2011/5/19	2011/5/21	血液・咽頭ぬぐい・尿	女	52y	不明	2.73	−	+	+		発熱(39.1℃)・全身の発疹・リンパ節腫脹・結膜充血	
19*	2011/5/21	2011/5/23	血液・咽頭ぬぐい・尿	女	20y	無	陽性 ***	−	+	−		発熱(38.6℃)・発疹・リンパ節腫脹	家族から
20	2011/5/30	2011/6/2	血液・咽頭ぬぐい・尿	男	25y	不明	−	−	+	+	2B	発熱(38℃)・発疹・コプリック斑・結膜充血	接触歴なし
21	2011/6/24	2011/6/25	血液・咽頭ぬぐい・尿	男	51y	記載	N.D.**	−	+	+		発熱(38.1℃)・発疹	
22	2011/7/4	2011/7/7	血液・咽頭ぬぐい・尿	男	15y	不明	2.91	−	−	−		発熱(39℃)・発疹・口内炎・コプリック斑疑い・結膜充血	接触歴なし
23*	2011/7/17	2011/7/17	血液・咽頭ぬぐい・尿	男	34y	無記載	N.D.**	−	+	−		発熱(37.7℃)・発疹	

*症例8、19および23では咽頭ぬぐい液からのウイルス分離陽性
**ND:検査せず
***コマーシャルラボの成績

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麻しん疑い症例検体から分離された風疹ウイルス—堺市

(Vol. 32 p. 257-258: 2011年9月号)

2012年の麻しん排除宣言に向けて、麻しん疑い症例から麻疹ウイルス遺伝子検出を積極的に行っているが、不検出例が続いている。そこで、臨床的に麻しんが疑われる症例の背景をウイルス感染に焦点を合わせて検索するため、培養細胞によるウイルス分離を試みている。その中で咽頭ぬぐい液を接種したVero-E6 にて細胞変性効果（CPE）様変化が見られた。この培養上清からヒトメタニューモウイルス（hMPV）、RSウイルス、パラインフルエンザウイルス、エンテロウイルスなどの遺伝子検出を試みたが、いずれも検出されなかった。ところが、大阪府内の風しん患者増加の情報から、検体培養上清の風疹ウイルス遺伝子検出を行ったところ、風疹ウイルスの増殖が確認されたので、分離株の遺伝子型について報告する。

材料：2011年１月～７月31日にかけて麻しん疑い症例で搬入された22症例の咽頭ぬぐい液22検体、尿20検体、合計42検体をhMPVの検出マニュアル（http://www.nih.go.jp/niid/reference/hMPV-manual.pdf）に準じて処理し、ウイルス分離検査に用いた。

ウイルス分離：分離細胞にはVero-E6を用いた。24穴マイクロプレートを使用し、フルシートになった時点でPBS 1mlで洗浄後、分離培養液を各穴に900μl、処理した検体を100μl接種し、34℃でCO_2インキュベーターにて培養し、細胞変性を観察した。分離培養液にはイーグルMEM 100mlに10% Fraction V 2mlを混和した液に、Acetylated Trypsin（1mg/ml）を100μl添加した。

ウイルス分離確認：５症例の咽頭ぬぐい液を接種したVero-E6にて、接種後５～７日目にエンテロウイルス様の小型で円形化した細胞が認められた。このCPE様変化は緩やかに進行し、正常な細胞が混在したままの状態であるため判別は困難であった。しかし、CPE様変化出現から７日経過したところで培養上清からRNA抽出を行い、10^{-1}～10^{-5}階乗希釈液の風疹ウイルス遺伝子検出を行った。各培養上清の抽出RNAは10^{-4}～10^{-5}希釈まで風疹ウイルス遺伝子が検出され、ウイルスが分離されたことが確認された。遺伝子検出にはE1P5/E1P8プライマーを用い病原体検出マニュアルに従った。

分離株遺伝子型別：分離株のE1遺伝子領域 739bpを用いた。遺伝子型別解析では、３株が2B型、残り２株はそれぞれ1E型と1j型に分類された。

風疹ウイルスが分離された５症例の臨床症状等と風疹ウイルス遺伝子型を示す（表１）。５症例のうち20代が３例、30代が２例、性別では４例が男性であった。30～50代男性の風疹抗体保有率が低いことが報告されているが、分離結果からはこれらの年代よりやや若い男性に感受性者がみられた。

大阪府の風しん届出患者数は2008年24人（全国294人）、2009年12人（全国147人）、2010年９人（全国90人）であったが、2011年は第31週までに41人（全国278人）と増加をみている（本号６ページ参照）。堺市においても、2008～2010年までは年間１～２人で推移していたが、2011年は第31週までで６人と増加している。遺伝子型では2B、1E、1jと少なくとも３遺伝子型の風疹ウイルスが感染に関与していた。風疹ウイルスの伝播は多様で、届出数以上の感染拡大の可能性が推測される。型別解析では堺市を含む大阪府内での遺伝子型は、検体数が少ないが、多くは2Bで、東南アジア等の流行国と一致した遺伝子型が検出された。麻しんと同様に輸入感染の可能性は否定できない。このような観点から、風疹ウイルスの分離、遺伝子型解析は風しん感染予防対策には重要である。

加えて、集団発生や先天性風しん症候群（CRS）の発生をなくすためにも、予防接種率のさらなる向上を推進しなければならない。

謝辞：風疹ウイルス遺伝子型別に当たり、ご指導いただきました国立感染症研究所ウイルス第三部の森嘉生先生に深

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表1. 風疹ウイルスが分離された症例

症例No.	年齢	性別	発症日	検体採取日	遺伝子型	発熱(℃)	発疹・紅斑	頸部リンパ節腫脹	ワクチン歴
1	39	男	3月18日	3月18日	1j	37.7	紅斑		不明
2	22	男	4月 1日	4月 3日	2B	38以上	有	有	無
3	20	女	5月20日	5月23日	2B	38.6	有	有	不明
4	34	男	7月17日	7月17日	2B	37.7	有		不明
5	20	男	7月27日	7月27日	1E	38.3	紅斑		不明

症例2:上海への渡航歴あり

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風疹ウイルスの遺伝子解析

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はじめに

世界保健機関（WHO）は風疹および先天性風疹症候群をワクチン接種によって制御すべき感染症の一つとして位置づけ、それに対する活動の一環として風疹および先天性風疹症候群に対する的確な実験室診断およびサーベイランスの整備をすすめている。特に遺伝子解析による病原体サーベイランスは、風疹ウイルス伝播の追跡を行う上で非常に重要な手法と考えられている。そのため、2004年に風疹ウイルスの系統学的な分類方法を定め、それに基づいた解析および報告を世界麻疹風疹実験室ネットワーク（The Global Measles and Rubella Laboratory Network; LabNet）に属する実験室に求めている1)。日本においては国立感染症研究所（感染研）ウイルス第三部がその任にあたっている。本稿ではWHOによって推奨されている風疹ウイルス遺伝子解析方法について解説する。

風疹ウイルスの構造

風疹ウイルスはトガウイルス科ルビウイルス属に分類される唯一のウイルスで、エンベロープを持つ直径約60nmの球形ウイルスである。ゲノムは通常9,672塩基からなるプラスセンス１本鎖RNAであり、そのG+C含有率は約70％と、これまでに配列の解析されたRNAウイルスでは最も高いことが知られている。ゲノムの５′側にはゲノムの転写複製に働く非構造蛋白質が、３′側にはウイルス粒子を形成する構造蛋白質（C、E2およびE1）がそれぞれコードされている。ウイルス粒子のエンベロープに存在するE2およびE1蛋白質はヘテロダイマーを形成し、ウイルスの感染性に関与する。特にE1蛋白質は中和抗体および赤血球凝集抑制抗体を誘導する主要な抗原となっている。

風疹ウイルスの遺伝子型分類法

構造蛋白質全領域（3,192塩基）のヌクレオチド配列解析により遺伝子型分類が行われ、現在のところ風疹ウイルスは二つのCladeに大きく分けられることが判明している2)。さらにClade1には10の遺伝子型（1a、1B、1C、1D、1E、1F、1G、1h、1i、1j）が、Clade2には３つの遺伝子型（2A、2B、2C）が存在する。Clade内のヌクレオチド変異率は５％以下であるが、Clade間では８～10％に達する。なお、このような差があっても風疹ウイルスの血清型は単一であり、ワクチンによる免疫を回避するウイルスの存在は知られていない。認定された遺伝子型はアルファベットの大文字で示され、暫定的な遺伝子型は小文字で示される。新しい遺伝子型が認定されるためには、少なくとも２つの参照となるウイルス分離株の詳細な解析が必要とされる。

一方で野外株の解析を簡易にするために、E1蛋白質領域の一部（8,731～9,469塩基領域；739塩基）が解析に必要な最小領域として用いられている1)。この領域の遺伝子配列を参照株のそれらと比較することにより、構造蛋白質全領域を解析した場合と同様の遺伝子型分類が可能となっている（図）。感染研ウイルス第三部第二室では、要望に応じてこの領域を増幅するプライマーセットの情報の提供をしている。

世界における流行株の遺伝子型

世界各国のLabNet実験室から報告された風疹ウイルスの遺伝子型情報を元に、過去から現在にかけてどの遺伝子型のウイルスが世界のどのような地域で流行していたかが解析されている。2005年１月～2010年５月までの期間で報告されたのは、13遺伝子型のうち９遺伝子型のウイルスである2)。報告のなかった４遺伝子型（1D、1F、1i、2A）のウイルスは、中国のワクチン株であるBRDII株（遺伝子型2A）を除いて消失したものと考えられている。一方、世界的な流行が認められているウイルスの遺伝子型は1E、1Gおよび2Bである。1E型ウイルスは、中東、ヨーロッパ、アフリカ、西太平洋地域で発生しており、特に中国では主要な遺伝子型ウイルスとなっている3)。1G型ウイルスはヨーロッパおよびアフリカ諸国で報告されている。2B型ウイルスは中東、ヨーロッパ、中南米、アフリカ、南～東南～東アジアで報告されている。その他の遺伝子型のウイルスの発生は、固有の地域に限定的である。

日本における流行ウイルスの遺伝子型の推移

日本においては1960年代後半に遺伝子型1aウイルスが流行していた。この型のウイルスが弱毒化され、現在日本にお

けるワクチン株として使用されている。1990年代には遺伝子型1Dウイルスが流行していたが、前述した通り現在では消失している。2000年代前半においては遺伝子型1jウイルスが主要なウイルスであった。2011年に遺伝子型1jウイルスの報告があったが、輸入症例であると推定されている4)。近年、日本では風疹の患者報告数が低く抑えられてきたが、2011年になって各地で流行が認められている。遺伝子型の判明している株のほとんどが遺伝子型1Eおよび2Bであり、これらのウイルスが流行の主体となっているものと思われる。2009年以前ではこれらの遺伝子型ウイルスでは南アジアからの輸入例での1例のみ（遺伝子型2B）しか報告されていなかったことから、近年の世界的な流行にともなって日本にも侵入し、急速に蔓延したものと考えられる。このように風疹ウイルスは数年～十数年ごとに遺伝子型が入れ替わりながら流行を繰り返してきている。

遺伝子型2Bウイルスはこれまで日本で流行してきたウイルスと塩基配列の置換が多いため、これまで用いられた遺伝子検出系がこの遺伝子型のウイルスを検出できるか確認をしておくことが重要であると考える。病原体検出マニュアル内の風疹の項目5) に記載されているRT-PCR用プライマーセットのうち、Primer A～Dは遺伝子型2Bウイルスに対しては検出感度が悪い可能性があることから、もう一つのプライマーセット（E1P5～E1P8）、もしくは感染研ウイルス第三部第二室で新規に設定したプライマーセット（要望に応じて情報提供可能）の使用を推奨する。

おわりに

WHO汎アメリカ地域およびヨーロッパ地域においては風疹および先天性風疹症候群の排除計画を積極的に推し進めている。日本の属するWHO西太平洋地域においても風疹および先天性風疹症候群の排除を目標に今後活動が強化されることが予想される。その際には麻疹排除計画の場合と同様に質の高いサーベイランス体制が必要となるであろう。麻疹の質の高いサーベイランスには、感染伝播が輸入症例に起源するか、あるいはその国に固有のものであるかを明らかにすることも含まれる6)。それに準じるならば、風疹ウイルスについても遺伝子解析を積極的に行い、国内外における風疹ウイルスの疫学的状況の把握を継続的に行っていくことが重要であると考えられる。

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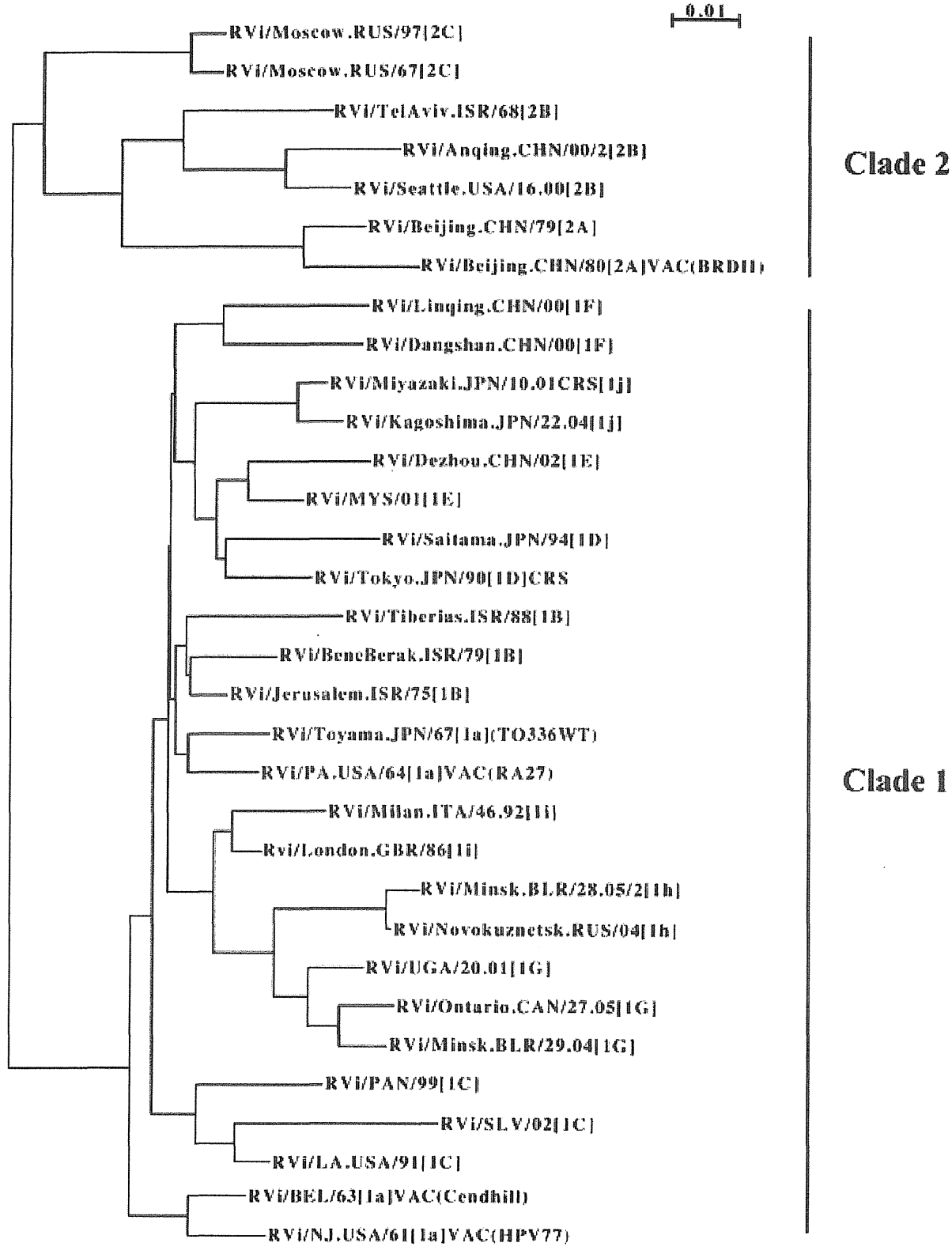

図. 風疹ウイルス参照株の最小解析領域(739bp)に基づく分子系統樹

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MRワクチン接種後、約４カ月を経て麻疹ワクチン株遺伝子が検出された症例―千葉県

(Vol. 32 p. 299-300: 2011年10月号)

症例は１歳７カ月男児で、2011年6月10日に咳が出現し、11日夜、38.5℃の発熱、耳、首に発疹が出現し、夜間救急診療所を受診し、コプリック斑が確認された。翌12日も発熱は持続し、かかりつけの医院を受診した。かかりつけ医において体幹から足および膝に広がる麻疹様発疹を認めたため、溶連菌感染症および麻疹を疑われたが、溶連菌検査キットで陰性のため、麻疹臨床診断例として保健所に届けられた。13日に発熱は40.0℃となった。14日には微熱となり、発疹は少しずつ消失した。17日、平熱、発疹は消失した。症例には免疫不全状態はなく、突発性発疹は満1歳の時点で罹患済みであり、正常経過であった。この男児は麻疹風疹混合ワクチン（MRワクチン：麻疹ワクチンはTanabe株）の接種を2011年1月24日に受けており、家族および男児が通所している保育所において、発症前の１カ月間に麻疹患者および麻疹ワクチン被接種者はいなかった。

６月12日採取の血液、咽頭ぬぐい液、尿について、麻疹ウイルスH遺伝子、N遺伝子に対するRT-PCRを実施したところ、咽頭ぬぐい液からのみH遺伝子、N遺伝子がともに検出された。N遺伝子486bpについて塩基配列を解析した結果、感染研分与の陽性コントロールAIK-C株とは異なり、接種を受けたMRワクチンに含まれるTanabe株と100％一致した。

麻疹IgM抗体は、６月12日、20日、７月４日のいずれも陰性であり、麻疹IgG抗体は、６月12日50.45 EIA価、20日53.33 EIA価、７月４日53.64 EIA価で、抗体価の変動はみられず、PA法による麻疹抗体価も変動はみられなかった。さらに、EBV、HSVの抗体検査において感染を示唆する結果は認められなかった。

また、急性期の６月12日の検体について、風疹ウイルス、パルボウイルスB19、ヒトヘルペスウイルス（HHV）6、HHV7、エンテロウイルス属、ライノウイルスについてPCRを実施したところ、咽頭ぬぐい液からHHV6およびライノウイルスの遺伝子を検出した。

さらに、麻疹ウイルスの感染が継続している可能性を疑い、７月４日に採取した血液、咽頭ぬぐい液を検査したが、麻疹ウイルス遺伝子は検出されなかった。検査結果の詳細については、表１に示した。

症例は、臨床症状から麻疹が強く疑われ、咽頭ぬぐい液から接種ワクチン株遺伝子が検出された。しかしながら麻疹抗体価は、発症時から約６週の間に３回得た血清において、すべてIgM抗体陰性、IgG抗体陽性であり、抗体価の変動も見られていない。また、HHV6、ライノウイルス遺伝子の検出およびEBV、HSVの抗体検査の結果も、今回の症状を直接説明できる結果ではないと考えた。

今回検出された麻疹ワクチン株遺伝子が患者の症状に関連するものなのか、何らかの要因とともに検出されてきたものなのかを明瞭にすることはできなかったが、抗体価の変動もなく、麻疹ウイルスに対する免疫応答はなかったといえる。また遺伝子の検出は一過性であり、ウイルス分離も陰性であったところから、持続感染や、他への感染の可能性等は否定できると考えられる。今後も、この症例の発疹出現時等にウイルスの検出を試みることは重要であろう。

なお今回、接種後約４カ月を経た麻疹様の発疹患者から、過去に接種を受けた麻疹ワクチン株と同一の遺伝子が検出されたが、麻疹ワクチン接種後の健常無症状者からのワクチンウイルス株遺伝子は、接種後２カ月以内までの末梢リンパ球から検出されたことが報告されている（Sonoda S, Nakayama T, J Med Virol 65: 381-387, 2001）。

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表1．麻疹様患者の検査

検体	採取月日	ウイルスおよび細菌検出						
		麻疹	風疹	B19	HHV6	HHV7	EV	溶連菌
リンパ球	6月12日	−	−	−	−	−	NT	NT
咽頭ぬぐい液		+	−	−	+	−	Rhinovirus	−
尿		−	−	−	NT	NT	NT	NT
血球	7月4日	−	−	−	−	−	NT	NT
咽頭ぬぐい液		−	−	−	−	−	Rhinovirus	−

検体	採取月日	抗体									
		麻疹IgM	麻疹IgG	麻疹PA	PVB19	HSVIgM	HSVIgG	EBIgM	EBIgG(VCA)	EBIgG(EADR)	EBIgG (EBNA)
血清Ⅰ	6月12日	−(0.704)	+(50.45)	256	−(0.22)	±(0.85)	−(0.5)	−(0.35)	−(0.3)	±(0.6)	−(0.2)
血清Ⅱ	6月20日	−(0.427)	+(53.33)	512	NT	NT	NT	NT	NT	NT	NT
血清Ⅲ	7月4日	−(0.319)	+(53.64)	512	NT	NT	NT	NT	NT	NT	NT

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Both RIG-I and MDA5 RNA Helicases Contribute to the Induction of Alpha/Beta Interferon in Measles Virus-Infected Human Cells[▽]

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Measles virus (MV), a member of the family *Paramyxoviridae*, is a nonsegmented negative-strand RNA virus. The RNA helicases retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) are differentially involved in the detection of cytoplasmic viral RNAs and induction of alpha/beta interferon (IFN-α/β). RIG-I is generally believed to play a major role in the recognition of paramyxoviruses, whereas many viruses of this family produce V proteins that can inhibit MDA5. To determine the individual roles of MDA5 and RIG-I in IFN induction after MV infection, small interfering RNA-mediated knockdown of MDA5 or RIG-I was performed in the human epithelial cell line H358, which is susceptible to wild-type MV isolates. The production of IFN-β mRNA in response to MV infection was greatly reduced in RIG-I knockdown clones compared to that in H358 cells, confirming the importance of RIG-I in the detection of MV. The IFN-β mRNA levels were also moderately reduced in MDA5 knockdown clones, even though these clones retained fully functional RIG-I. A V protein-deficient recombinant MV (MVΔV) induced higher amounts of IFN-β mRNA at the early stage of infection in H358 cells compared to the parental virus. The reductions in the IFN-β mRNA levels in RIG-I knockdown clones were less pronounced after infection with MVΔV than after infection with the parental virus. Taken together, the present results indicate that RIG-I and MDA5 both contribute to the recognition of MV and that the V protein promotes MV growth at least partly by inhibiting the MDA5-mediated IFN responses.

Alpha/beta interferons (IFN-α/β) play central roles in the host defense against viral infections (42). Pattern recognition receptors are the host molecules that detect pathogen-associated molecular patterns and activate the innate immune responses that operate at the early stage of infections (1). Toll-like receptor 3 (TLR3) and TLR7 recognize viral RNAs in the endosome and induce the production of various cytokines, including IFNs. In the cytoplasm of virus-infected cells, two RNA helicases, melanoma differentiation-associated gene 5 (MDA5) and retinoic acid-inducible gene I (RIG-I), are involved in virus recognition and IFN induction (21, 58, 59). Studies have shown that MDA5 recognizes long double-stranded RNAs (dsRNAs) (22), which are produced in cells infected with picornaviruses and reoviruses (23, 28), while RIG-I detects single-stranded RNAs with 5′-triphosphate (19, 37) and short dsRNAs (22), which are found in cells infected with a variety of RNA viruses. Therefore, it is generally believed that RIG-I plays a major role in the recognition of many RNA viruses, including paramyxoviruses (23, 28), whereas MDA5 only acts as an RNA sensor for certain RNA viruses.

Measles is a febrile acute infectious disease that remains a major cause of child deaths worldwide, especially in developing countries (7). Measles virus (MV) is a member of the genus *Morbillivirus* in the family *Paramyxoviridae*. The MV genome possesses six genes, which encode N (nucleocapsid), P (phospho-), M (matrix), F (fusion), H (hemagglutinin), and L (large) proteins, respectively (16). The P gene encodes two additional proteins, the V and C proteins (5, 10). During transcription of the P gene, an additional guanine residue may be inserted at a specific site in the nascent transcript via the recognition of an editing motif, producing the V mRNA (10). Consequently, the V protein shares the N-terminal 231 amino acid residues with the P protein but has a unique C-terminal region with 68 amino acid residues. The C protein is translated from the P and V mRNAs using an alternative reading frame (5). Although the V and C proteins are dispensable for MV growth in some cultured cells (40, 45), they promote viral replication by circumventing the host innate immune responses (11, 31, 53) and are associated with MV virulence in vivo (12, 35, 53, 54). The V protein blocks signal transduction in response to IFN-α/β (8, 13, 14, 32, 34, 41, 51, 57) and inhibits TLR7- and TLR9-mediated IFN-α/β production in human plasmacytoid dendritic cells by binding to IκB kinase α and IFN regulatory factor (IRF) 7 (36). Furthermore, the V proteins of many paramyxoviruses, including MV, bind to MDA5 and inhibit its function (2). Therefore, the role of MDA5 in the recognition of paramyxoviruses may have been underestimated in previous studies.

In the present study, we generated RIG-I and MDA5 knockdown human cells and a V protein-deficient recombinant MV (MVΔV) and examined the roles of RIG-I and MDA5 in the detection of MV and the resulting IFN induction. Our results indicated that these antiviral RNA helicase proteins both con-

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tribute to IFN induction in response to MV infection and that the V protein promotes MV growth at least partly by inhibiting the MDA5-mediated response in infected cells.

MATERIALS AND METHODS

Plasmid constructions. Using the pBAsi-hU6 pur DNA vector (Takara Bio, Inc., Shiga, Japan), we constructed three plasmids—pBAsi-MDA5, pBAsi-RIG-I, and pBAsi-luciferase—that generated stem-loop type small interfering RNAs (siRNAs). The siRNAs from pBAsi-MDA5, pBAsi-RIG-I, and pBAsi-luciferase targeted the mRNAs of human MDA5 and RIG-I and firefly luciferase, respectively. The target sequences for the human MDA5 and RIG-I and firefly luciferase mRNAs were 5′-GGAGAAUAACUCAUCAGAAUC-3′, 5′-GCCAGAAUCUUAGUGAGAAUU-3′, and 5′-GCCCGCGAACGACAUUUAUAA-3′, respectively.

The plasmid p(+)MV323-EGFP was derived from p(+)MV323, which encoded the full-length antigenomic cDNA of the virulent IC-B strain of MV, and contained an additional transcriptional unit for enhanced green fluorescent protein (EGFP) (17, 50). The plasmid p(+)MVΔV-EGFP was generated by introducing four nucleotide substitutions into the region corresponding to the RNA editing motif of the P gene of p(+)MV323-EGFP. All four substitutions were synonymous in the reading frame of the P protein. Recombinant MVs generated from p(+)MV323-EGFP and p(+)MVΔV-EGFP were designated wild-type (wt) MV and MVΔV, respectively, in the present study.

Cells and viruses. H358 (49), VV5-4 (3), and B95a (24) cells were maintained in RPMI 1640 medium (ICN Biomedicals, Aurora, OH) supplemented with 7.5% fetal bovine serum (FBS). To generate cells constitutively expressing the siRNAs targeting the mRNAs of human MDA5 and RIG-I and firefly luciferase, H358 cells cultured in 3.5-cm dishes were transfected with 5 μg of pBAsi-MDA5, pBAsi-RIG-I, or pBAsi-luciferase using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA), according to the manufacturer's instructions. At 36 h posttransfection, the cells were harvested, transferred onto 10-cm dishes, and selected in RPMI 1640 medium supplemented with 7.5% FBS and 1 μg of puromycin/ml. After 3 weeks of culture, the puromycin-resistant clones formed colonies on the 10-cm dishes, and three clones each were isolated from the pBAsi-MDA5-transfected cells (clones M1, M2, and M3) and pBAsi-RIG-I-transfected cells (clones R1, R2, and R3). One clone was also isolated from the pBAsi-luciferase-transfected cells. These clones were propagated for further analyses. Vero cells constitutively expressing human signaling lymphocyte activation molecule (SLAM) (Vero/hSLAM) (33) were maintained in Dulbecco's modified Eagle medium (ICN Biomedicals) supplemented with 7.5% FBS and 500 μg of Geneticin (G418; Nacalai Tesque, Tokyo, Japan)/ml. Recombinant MVs were generated in VV5-4 cells from the cloned cDNAs and propagated as described previously (29, 47).

Reagents and antibodies. A fusion blocking peptide (FBP), Z-D-Phe-Phe-Gly, was purchased from Peptide Institute (Osaka, Japan) (43). IFN-αA/D was purchased from Sigma-Aldrich (St. Louis, MO). A serum sample from a patient with subacute sclerosing panencephalitis (56) was kindly provided by M. B. A. Oldstone, The Scripps Research Institute, La Jolla, CA, and used to detect the MV N protein. Rabbit polyclonal antibodies against the MV P and V proteins and a mouse monoclonal antibody against the MV C protein (clone 2D10) were described previously (30, 31). Rabbit polyclonal antibodies against MDA5 and RIG-I were purchased from ProSci., Inc. (Poway, CA). A rabbit polyclonal antibody against IRF3 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A mouse monoclonal antibody against human actin (clone C2) and a polyclonal antibody against IFN-induced protein with tetratricopeptide repeats 1 (ISG56) were purchased from Santa Cruz Biotechnology and Abnova Corp. (Taipei City, Taiwan), respectively. Horseradish peroxidase-conjugated donkey anti-rabbit and sheep anti-mouse immunoglobulin antibodies were purchased from GE Healthcare (Piscataway, NJ). A horseradish peroxidase-conjugated goat anti-human immunoglobulin antibody was purchased from EY Laboratories (San Mateo, CA).

Virus titration. Monolayers of Vero/hSLAM cells on 12-well plates were incubated with serially diluted virus samples for 1 h at 37°C, washed with phosphate-buffered saline, and overlaid with Dulbecco's modified Eagle medium containing 2% FBS and 1.5% methylcellulose 4000 (Wako Pure Chemical Industries, Osaka, Japan). At 5 days after infection, the numbers of PFU were determined.

Western blot analysis. After being washed with phosphate-buffered saline, cells were lysed with a buffer (150 mM NaCl, 10 mM Tris-HCl [pH 7.4], 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]), and the polypeptides in the lysates were reduced by heating in the presence of β-mercaptoethanol (Nacalai Tesque). For the detection of IRF3 dimers, cells were lysed with a different lysis buffer (150 mM NaCl, 50 mM Tris-HCl [pH 8.0], 1% NP-40) (20) supplemented with serine/threonine phosphatase inhibitor (Sigma-Aldrich), tyrosine phosphatase inhibitor (Sigma-Aldrich), and protease inhibitor cocktails (Sigma-Aldrich), and the polypeptides in the lysates were not reduced. The polypeptides were separated by SDS-polyacrylamide gel electrophoresis (PAGE), except for those prepared to detect IRF3 dimers, which were separated by native PAGE (20). The separated polypeptides were electroblotted onto polyvinylidene difluoride (PVDF) membranes (Hybond-P; GE Healthcare). The PVDF membranes were incubated with appropriate primary antibodies for 16 h at 4°C, washed three times with Tris-buffered saline containing 0.05% Tween 20 (TBST), and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. After three washes with TBST, the PVDF membranes were treated with ECL Plus reagents (GE Healthcare), and chemiluminescent signals were detected and visualized using a VersaDoc 3000 Imager (Bio-Rad, Hercules, CA). The relative amounts of the proteins were determined by densitometry using the Quantity One software (Bio-Rad).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNAs were extracted and purified from cells using TRIzol reagent (Invitrogen Life Technologies), treated with RQ1 DNase (Promega, Madison, WI), and reverse transcribed into cDNAs by using a PrimeScript RT reagent kit and an oligo(dT) primer (Takara Bio, Inc.), according to the manufacturer's instructions. The amounts of cDNAs for the viral mRNAs and host β-actin and IFN-β mRNAs were quantified by using SYBR Premix Ex *Taq* II (Takara Bio, Inc.) and a LightCycler (Roche Diagnostics, Indianapolis, IN) as described previously (31, 48).

Transfection with MV leader RNA. MV leader RNA was synthesized in vitro by using a MEGAshortscript kit (Ambion, Austin, TX) according to the manufacturer's instructions, and the in vitro transcription products were purified by using TRIzol reagent as previously described (31). Monolayers of cells on six-well plates were transfected with 1 μg of MV leader RNA by using Lipofectamine 2000. Cellular RNAs were collected at 6 h posttransfection.

RESULTS

Generation of MDA5 or RIG-I knockdown cells. For the present study, we used the human epithelial cell line H358, which is susceptible to clinical MV isolates via an as-yet-unidentified receptor (49). Our preliminary results indicated that the V protein may play an important role in MV growth in H358 cells, since MV lacking the V protein exhibited reduced growth in this cell line, but not in other cell lines (see below). To evaluate the individual contributions of MDA5 and RIG-I to the induction of IFN-α/β after MV infection, we produced H358 cells that constitutively expressed siRNAs targeting the MDA5 and RIG-I mRNAs. Three MDA5 knockdown clones and three RIG-I knockdown clones were selected to minimize the effects of clonal variations. The expression levels of MDA5 and RIG-I were examined by Western blot analysis in these clones at 24 h after treatment with IFN-αA/D (Fig. 1), since MDA5 and RIG-I were only expressed at low levels without IFN treatment. The signal intensities for MDA5 and RIG-I were quantified and normalized by that for β-actin. All three MDA5 knockdown clones (M1, M2, and M3) expressed MDA5 at levels of <5% compared to the parental H358 cells, whereas the RIG-I knockdown clones (R1, R2, and R3) expressed RIG-I at levels of 5 to 35% compared to the parental H358 cells.

IFN-β mRNA expression in MDA5 and RIG-I knockdown clones after MV infection. IFN-β mRNA expression was examined by RT-qPCR in the MDA5 and RIG-I knockdown clones, as well as in H358 cells after infection with wt MV. A clone expressing a nontargeting luciferase siRNA was also included as a control for the analysis. The IFN-β mRNA levels were very low in all cells at 24 h after infection (data not shown). At 48 h after infection, all three RIG-I knockdown

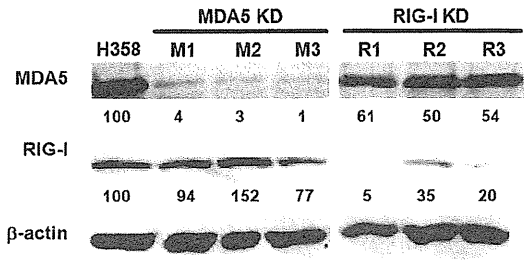


FIG. 1. Expression of MDA5 and RIG-I in H358 cells and knockdown clones. The expression levels of MDA5 and RIG-I in the parental H358 cells, MDA5 knockdown clones (MDA5 KD: M1, M2, and M3), and RIG-I knockdown clones (RIG-I KD: R1, R2, and R3) were examined by Western blot analysis at 24 h after treatment with IFN-αA/D. β-actin was evaluated as an internal control. Indicated blow each band are the signal intensities of MDA5 and RIG-I, which were normalized by that of β-actin. The value of the parental H358 cells was set to 100%.

clones produced IFN-β mRNA at levels of <5% compared to the parental H358 cells (Fig. 2A), in agreement with previous findings that RIG-I is a chief cytoplasmic RNA sensor for the detection of paramyxovirus infections (23, 28). H358 cells inoculated with wt MV in the presence of the FBP or with UV-inactivated wt MV expressed as low levels of IFN-β mRNA as the mock-infected cells, confirming that MV replication in the cytoplasm was required for the induction of IFN-β mRNA expression in H358 cells. Furthermore, the control H358 cells expressing a nontargeting siRNA produced almost the same amount of IFN-β mRNA as the parental H358 cells after infection with wt MV, indicating that the presence of siRNA per se does not affect the expression of IFN-β mRNA.

If RIG-I is largely responsible for detecting MV RNA, knockdown of MDA5 should not affect the expression levels of IFN-β mRNA in H358 cells. However, expression of IFN-β mRNA was reduced by 60 to 80% in the three MDA5 knockdown clones (Fig. 2A). To confirm that RIG-I was functioning properly in these MDA5 knockdown clones, the clones were treated with MV leader RNA transcribed in vitro, which has been shown to induce IFN-α/β via RIG-I (38). All three MDA5 knockdown clones, as well as the control clone expressing a nontargeting siRNA, expressed similar or even higher levels of IFN-β mRNA in response to the MV leader RNA compared to the parental H358 cells (Fig. 2B). As expected, the RIG-I knockdown clones exhibited reduced expression of IFN-β mRNA in response to the MV leader RNA. These results indicate that not only RIG-I but also MDA5 is involved in IFN induction after MV infection.

Generation and properties of a recombinant MV lacking the V protein. Many previous studies may have failed to demonstrate the contribution of MDA5 to IFN induction because paramyxoviruses produce V proteins that bind to MDA5, thereby inhibiting its function. To examine the activity of MDA5 in the absence of the V protein, we generated a recombinant MV incapable of producing the V protein (MVΔV). To this end, four point mutations were introduced into the P gene editing motif of wt MV, such that they were synonymous in the reading frame of the P protein (Fig. 3A). MVΔV grew efficiently in SLAM-positive B95a cells (data not

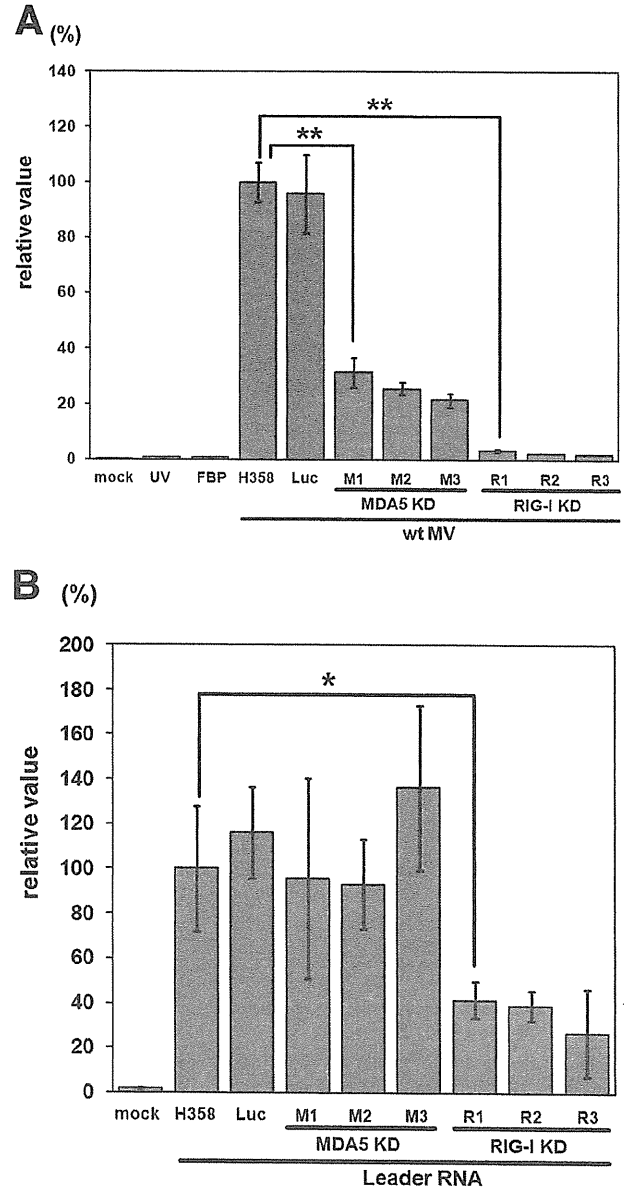


FIG. 2. IFN-β mRNA expression in H358 cells and knockdown clones after MV infection or transfection with MV leader RNA. (A) The parental H358 cells, the clone expressing a nontargeting luciferase siRNA (Luc), MDA5 knockdown clones (MDA5 KD: M1, M2 and M3) and RIG-I knockdown clones (RIG-I KD: R1, R2 and R3) were infected with wt MV at a multiplicity of infection (MOI) of 0.5. H358 cells were also infected with UV-inactivated wt MV (UV), treated with an FBP before wt MV infection (FBP), or mock infected (mock). Total RNAs were extracted from the cells at 48 h after infection, and the IFN-β mRNA levels were quantified by RT-qPCR. (B) H358 cells, the clone expressing a luciferase siRNA, and MDA5 and RIG-I knockdown clones were transfected with in vitro-transcribed MV leader RNA. H358 cells were also mock transfected (mock). Total RNAs were extracted from the cells at 6 h posttransfection, and the IFN-β mRNA levels were quantified by RT-qPCR. All data were normalized by the corresponding β-actin mRNA levels in the respective cells. The mean value in the parental H358 cells infected with wt MV (A) or transfected with MV leader RNA (B) was set to 100%. The data represent the means ± the standard deviations of triplicate samples. *, $P < 0.05$; **, $P < 0.01$ (significant differences based on a t test).

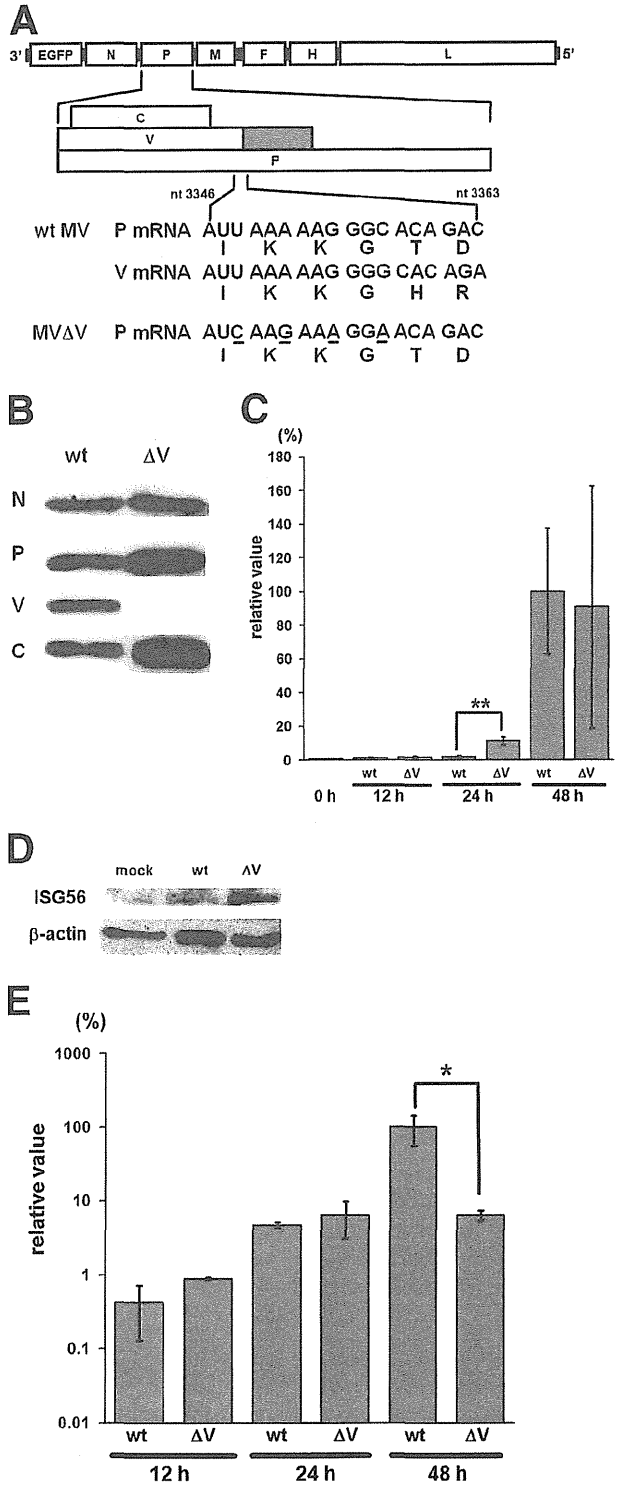
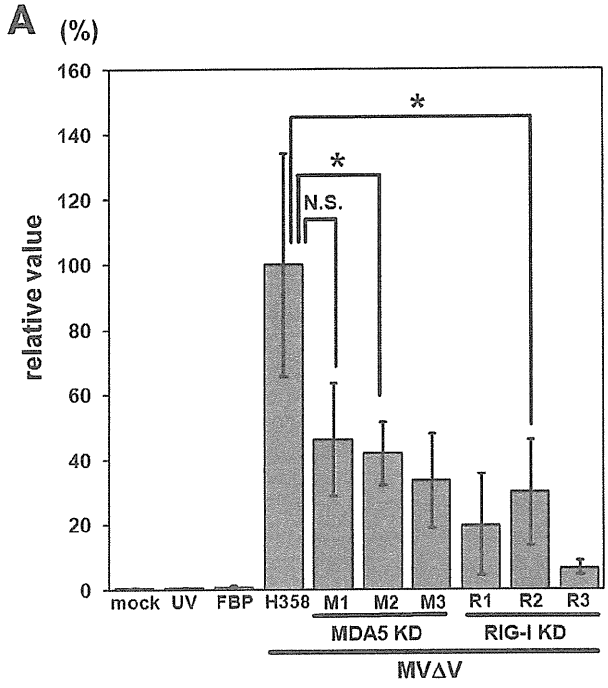


FIG. 3. Generation and properties of the V protein-deficient MV (MVΔV). (A) Diagram of the MV genome indicating the locations of the introduced mutations to generate MVΔV. Underlines indicate the mutated nucleotides. The predicted amino acids are shown below the trinucleotide codons. nt, nucleotide. (B) Protein synthesis in MV-infected cells. B95a cells were infected with wt MV or MVΔV at an MOI of 0.01. At 36 h after infection, viral proteins (N, P, V, and C) in the infected cells were detected by Western blot analysis. (C) IFN-β mRNA levels in H358 cells infected with wt MV or MVΔV at an MOI of 0.5. At 12, 24, and 48 h after infection, total RNA was extracted from the MV-infected cells, and the IFN-β mRNA levels were quantified by RT-qPCR. The data were normalized by the β-actin mRNA levels, and the mean value in wt MV-infected cells at 48 h after infection was set to 100%. The data represent the means ± the standard deviations of triplicate samples. The IFN-β mRNA level in uninfected cells is also shown (0 h). **, $P < 0.01$ (significant difference based on a t test). (D) Induction of ISG56 in MV-infected cells. H358 cells were mock infected or infected with wt MV or MVΔV at an MOI of 0.5. At 24 h after infection, the ISG56 levels in the infected cells were detected by Western blot analysis. β-Actin was analyzed as an internal control. (E) N mRNA levels in H358 cells infected with wt MV or MVΔV at an MOI of 0.5. At 12, 24, and 48 h after infection, total RNA was extracted from MV-infected cells, and the N mRNA levels were quantified by RT-qPCR. The data were normalized by the β-actin mRNA levels, and the mean value in wt MV-infected cells at 48 h after infection was set to 100%. *, $P < 0.05$ (significant difference based on a t test).

shown). cDNAs of the P and V mRNAs from MVΔV-infected B95a cells were amplified by PCR, and the PCR products were cloned and sequenced to determine the frequency of the RNA editing. Although 42% (20/48) of the PCR products from wt MV-infected cells had one or more additional guanine residues at the editing site, none (0/45) of the PCR products from MVΔV-infected cells had any additional nucleotides. Western blot analysis further confirmed that the P and C proteins, but not the V protein, were produced in MVΔV-infected B95a cells (Fig. 3B).

In H358 cells, IFN-β mRNA production was hardly increased above the background (uninfected) level at 12 and 24 h after infection with wt MV (Fig. 3C). MVΔV induced ~6 times more IFN-β mRNA in H358 cells at 24 h after infection compared to wt MV. Similarly, MVΔV induced a larger amount of the IRF3-activated protein ISG56 at 24 h after infection compared to wt MV (Fig. 3D). At 12 and 24 h after infection, the amounts of the N mRNA determined by RT-qPCR were comparable between wt MV- and MVΔV-infected H358 cells (Fig. 3E). Therefore, a difference in the amounts of viral RNAs produced was unlikely to be responsible for the difference in the amounts of IFN-β mRNA and ISG56 induced. Taken together, these results suggest that the absence of the V protein led to enhanced activity of MDA5 in MVΔV-infected cells, which in turn caused higher inductions of IFN-β and ISG56. The amount of the N mRNA at 48 h after MVΔV infection was ~10-fold lower than that after wt MV infection (Fig. 3E), which was probably due to the higher levels of IFN induced at the early stage of infection (24 h after infection) in MVΔV-infected cells, resulting in the inhibition of virus replication. This reduction of MV replication (and therefore viral RNA synthesis) at later times in MVΔV-infected cells may also explain why at 48 h after infection, there is little difference in the amount of IFN produced between wt MV- and MVΔV-infected cells (Fig. 3C).

Infection of MDA5 and RIG-I knockdown cells with MVΔV. Next, we infected MDA5 and RIG-I knockdown clones, as well as H358 cells, with MVΔV. Detection of MV infection appeared to be suppressed in MVΔV-infected MDA5 knockdown clones, as evidenced by decreases in the production of



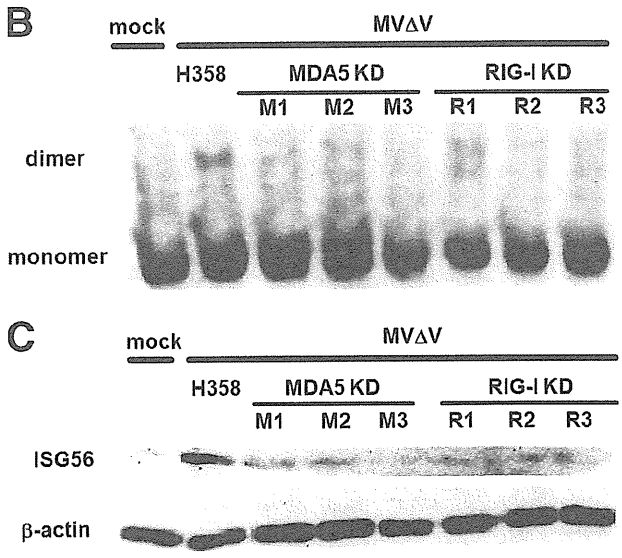


FIG. 4. Inductions of IFN-β mRNA and ISG56 and dimerization of IRF3 in MVΔV-infected cells. (A) The parental H358 cells, MDA5 knockdown clones (MDA5 KD: M1, M2, and M3) and RIG-I knockdown clones (RIG-I KD: R1, R2, and R3) were infected with MVΔV at an MOI of 0.5. H358 cells were also infected with UV-inactivated MVΔV (UV), treated with an FBP before MVΔV infection (FBP), or mock infected (mock). Total RNAs were extracted from the cells at 24 h after infection, and the IFN-β mRNA levels were quantified by RT-qPCR. The data were normalized by the β-actin mRNA levels, and the mean value in MVΔV-infected H358 cells was set to 100%. *, $P < 0.05$ (significant difference based on a t test). N.S., not significant. (B and C) H358 cells and MDA5 and RIG-I knockdown clones were infected with MVΔV at an MOI of 0.5. H358 cells were also mock infected. At 24 h after infection, the monomeric and dimeric forms of IRF3 in the cells were examined by native PAGE and Western blot analysis (B), and the ISG56 levels in the cells were detected by SDS-PAGE and Western blot analysis (C). β-Actin was examined as an internal control.

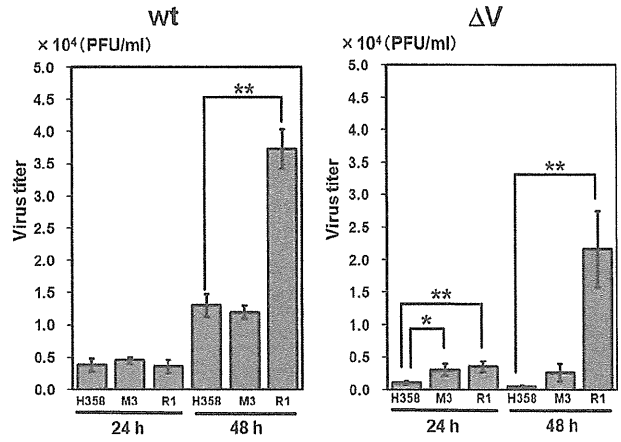


FIG. 5. Growth kinetics of wt MV and MVΔV in H358 cells and knockdown clones. The parental H358 cells, an MDA5 knockdown clone (M3) and an RIG-I knockdown clone (R1) were infected with wt MV or MVΔV at an MOI of 0.5. At 24 and 48 h after infection, the cells were harvested, together with the culture media, and the virus titers were determined by plaque assays. The data represent the means ± the standard deviations of triplicate samples. *, $P < 0.05$; **, $P < 0.01$ (significant differences based on a t test).

IFN-β mRNA (Fig. 4A), the dimerization of IRF3 (Fig. 4B), and the production of ISG56 (Fig. 4C). As expected, these three parameters were also suppressed in RIG-I knockdown clones (Fig. 4A to C). In control experiments, all of the knockdown clones were found to exhibit IRF3 dimer formation and express ISG56 after transfection with Cardif/VISA/MAVS/IPS-1, the common adaptor molecule downstream of MDA5 and RIG-I (42; data not shown). These findings indicate that MDA5 does indeed contribute to the detection of MV and resulting induction of IFN-β and that MDA5 and RIG-I are both required for H358 cells to fully produce IRF3-activated IFN-β and ISG56 after MVΔV infection. It was noted that significant levels of IFN-β mRNA were produced in MVΔV-infected RIG-I knockdown clones (Fig. 4A), unlike the case for RIG-I knockdown clones infected with wt MV, in which the production of IFN-β mRNA was negligible compared to wt MV-infected parental H358 cells (Fig. 2A). These observations may further support the role of MDA5 in IFN induction after MV infection, which is only revealed in the absence of the V protein.

Growth of wt MV and MVΔV in knockdown clones. The growth kinetics of wt MV and MVΔV were examined in H358 cells, an MDA5 knockdown clone (M3), and a RIG-I knockdown clone (R1) (Fig. 5). At 24 h after infection when the productions of IFN-β mRNA were still very limited (Fig. 3C), there was little difference in the growth of wt MV among H358, M3, and R1 cells. At 48 h after infection, the growth of wt MV was accelerated in R1 cells, but not in M3 cells, compared to H358 cells. Virus titers may be determined by the balance between the speed of virus replication and antiviral action of IFN. Thus, the reduction of IFN-β mRNA found in M3 cells may not have been sufficient to enhance wt MV replication at 48 h after infection, unlike that in R1 cells. On the other hand, the growth of MVΔV was strongly suppressed in H358 cells. Growth retardation of MVΔV may be expected to be compen-

sated for in MDA5 knockdown cells, and MVΔV did indeed grow better in M3 cells than in H358 cells. However, the growth of MVΔV in M3 cells was still less efficient than that of wt MV in H358 cells. Furthermore, MVΔV growth was greatly enhanced in R1 cells compared to H358 and M3 cells at 48 h after infection, reconfirming the primary importance of RIG-I in the detection of MV and subsequent IFN production.

DISCUSSION

In the present study, we have shown that RIG-I and MDA5 are both involved in the detection of MV in human cells and that the V protein plays an important role in MV growth by inhibiting the MDA5-mediated induction of the host IFN responses.

Previous studies using embryonic fibroblasts from MDA5$^{-/-}$ or RIG-I$^{-/-}$ knockout mice in vitro, as well as these knockout mice in vivo, have shown that these RNA helicases have differential roles in the recognition of different viruses. Specifically, RIG-I is essential for the detection of many RNA viruses including paramyxoviruses, orthomyxoviruses, rhabdoviruses and flaviviruses, while MDA5 is critical for the detection of picornaviruses (15, 23, 28). Interestingly, dengue virus type 2, a flavivirus, and reoviruses were shown to induce innate immune responses in embryonic fibroblasts obtained from either MDA5$^{-/-}$ or RIG-I$^{-/-}$ mice, indicating that they trigger both RIG-I- and MDA5-dependent responses (28).

Although the above generalization may be made, the data are more complicated for individual viruses. In studies with knockout mice, IFN production was significantly diminished in RIG-I$^{-/-}$ cells, but not in MDA5$^{-/-}$ cells, after infection with paramyxoviruses such as Sendai virus (SeV), including that lacking the V protein, Newcastle disease virus (NDV), and respiratory syncytial virus (23, 28). On the other hand, Yoneyama et al. (58) demonstrated that expression of IFN mRNA after NDV infection was inhibited in mouse fibroblast L929 cells by siRNA-mediated knockdown of either RIG-I or MDA5. Studies with the monoclonal antibody J2, which specifically recognizes dsRNAs of more than 40 bp in length (55), detected dsRNAs in NDV-infected cells (52) but not in SeV-infected cells (52, 55).

In the case of MV, infection was found to trigger IFN responses in human Huh7 cells but not in their derivative Huh7.5 cells with nonfunctional RIG-I, although the MDA5 pathway was intact in both cell lines (38). Another study suggested that MDA5 is involved in MV-induced expression of IFN-β since forced expression of MDA5, but not RIG-I or TLR3, led to enhanced IFN-β promoter activity in MV-infected human A549 cells (6). Shingai et al. (46) reported that vaccine strains of MV contain defective interfering (DI) particles, which are responsible for IFN-β induction. These authors also showed that both RIG-I and MDA5 detect the stem-loop structure of DI RNAs, whereas only RIG-I senses DI RNAs with 5′-triphosphate. Therefore, differences in the cell types (knockout mouse-derived cells versus cultured cells) and assay systems used may have led to various conclusions regarding the relative roles of RIG-I and MDA5 in the detection of different paramyxoviruses. Furthermore, although all paramyxoviruses have a common life cycle including genome replication and transcription (25), host cells may recognize different paramyxoviruses in different manners.

To determine the individual roles of MDA5 and RIG-I in IFN induction after MV infection, we established human epithelial H358 cells constitutively expressing siRNAs targeting the MDA5 and RIG-I mRNAs. Even in the RIG-I knockdown clones that still expressed considerable levels of RIG-I (clones R2 and R3), induction of IFN-β mRNA after MV infection was almost completely blocked, indicating that RIG-I plays a major role in the recognition of MV. The results are consistent with those obtained using Huh7 and Huh7.5 cells (38), as well as with the general notion regarding the importance of RIG-I in the detection of paramyxoviruses. However, our data further showed that MDA5 is also involved in the detection of MV, since IFN-β mRNA production in MDA5 knockdown clones was reduced compared to that in the parental H358 cells. This observation appears to contradict the finding that RIG-I knockdown clones (expressing normal levels of MDA5) hardly produced IFN-β mRNA after MV infection. Without RIG-I, MDA5-mediated signaling probably does not function sufficiently to induce IFN in the presence of the antagonizing V protein.

To evaluate the contribution of MDA5 to IFN induction in the absence of the V protein, a V protein-deficient MV (MVΔV) was generated. IFN-β mRNA and ISG56 were induced at higher levels in MVΔV-infected cells than in wt MV-infected cells at 24 h after infection. Similar enhancement of IFN production has also been reported for the paramyxovirus simian virus 5 with a defective V protein (18). Activation of IRF3 and production of IFN-β mRNA and ISG56 in response to MVΔV infection were reduced in MDA5 knockdown cells compared to the parental H358 cells. Importantly, significant levels of IFN-β mRNA were produced in RIG-I knockdown cells infected with MVΔV, unlike the case for those infected with wt MV. Furthermore, the growth of MVΔV, but not that of wt MV, was enhanced in MDA5 knockdown cells. All of these results indicate that MDA5 is indeed involved in the recognition of MV and that the V protein is required for MV to inhibit the MDA5-mediated IFN induction.

MDA5 and RIG-I appear to recognize different RNA structures (19, 22, 37, 44). Leader RNA, a 5′-triphosphate ended single-stranded RNA, is produced during the transcription of MV and other paramyxoviruses (9, 26), and in vitro-synthesized MV leader RNA was found to induce IFN through RIG-I (38). Therefore, RIG-I probably detects leader RNA in MV-infected cells and induces IFN production. Using the dsRNA-specific antibody J2 (55), we tried to detect intracellular dsRNA, which is the ligand for MDA5 (22). Although dsRNA was not found in wt MV- or MVΔV-infected Vero/hSLAM cells, it was clearly detected in MVΔC-infected cells (data not shown). A recent study also reported that dsRNA exists in cells infected with a C protein-deficient SeV, but not in SeV-infected cells (52). Therefore, the C proteins of both SeV and MV may act to limit the generation of intracellular dsRNA. It is likely that dsRNA is generated at a low level in wt MV- or MVΔV-infected cells, although it cannot be detected by the J2 antibody in the presence of the C protein. Alternatively, unknown structural motifs may exist in MV RNA that can activate MDA5.

The V protein interferes with multiple steps of host antiviral

responses such as IFN-α/β induction (36, 39) and IFN-α/β-mediated signal transduction (32, 34, 51, 57). Since the MV P protein can inhibit IFN signaling like the V protein (13, 32), the raison d'être for the MV V protein may largely reside in its blockade of MDA5-mediated IFN induction. However, the significance of this V protein function may vary among different cell types, because MV lacking the V protein grows comparably to the parental virus in B95a cells (present study) but not in H358 cells (present study) and rhesus monkeys (12). In the present study, knockdown of MDA5 weakened IFN induction in H358 cells after infection with wt MV or MVΔV. On the other hand, the growth defect of MVΔV was not completely compensated for in MDA5 knockdown H358 cells, indicating that the V protein may contribute to efficient MV growth partly by inhibiting MDA5-mediated IFN induction and partly by other mechanisms.

Given the importance of RIG-I in the recognition of paramyxoviruses, including MV, inhibition of RIG-I, rather than MDA5, may be a better strategy for MV to achieve efficient growth. Indeed, some paramyxoviruses block RIG-I-mediated IFN-α/β induction (4, 27). Strong suppression of innate immunity through blockade of RIG-I may increase MV growth but possibly kill the host too quickly for the virus to spread to other hosts. Blockade of MDA5 may be more advantageous for MV survival, by allowing both sufficient growth within a host and efficient spread to different hosts.

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Epithelial-Mesenchymal Transition Abolishes the Susceptibility of Polarized Epithelial Cell Lines to Measles Virus*[S]

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Measles virus (MV), an enveloped negative-strand RNA virus, remains a major cause of morbidity and mortality in developing countries. MV predominantly infects immune cells by using signaling lymphocyte activation molecule (SLAM; also called CD150) as a receptor, but it also infects polarized epithelial cells, forming tight junctions in a SLAM-independent manner. Although the ability of MV to infect polarized epithelial cells is thought to be important for its transmission, the epithelial cell receptor for MV has not been identified. A transcriptional repressor, Snail, induces epithelial-mesenchymal transition (EMT), in which epithelial cells lose epithelial cell phenotypes, such as adherens and tight junctions. In this study, EMT was induced by expressing Snail in a lung adenocarcinoma cell line, II-18, which is highly susceptible to wild-type MV. Snail-expressing II-18 cells lost adherens and tight junctions. Microarray analysis confirmed the induction of EMT in II-18 cells and suggested a novel function of Snail in protein degradation and distribution. Importantly, wild-type MV no longer entered EMT-induced II-18 cells, suggesting that the epithelial cell receptor is down-regulated by the induction of EMT. Other polarized cell lines, NCI-H358 and HT-29, also lost susceptibility to wild-type MV when EMT was induced. However, the complete formation of tight junctions rather reduced MV entry into HT-29 cells. Taken together, these data suggest that the unidentified epithelial cell receptor for MV is involved in the formation of epithelial intercellular junctions.

Measles is an acute febrile disease transmitted via aerosol droplets and remains a major cause of infant death in developing countries (1). Measles virus (MV),[2] the causative agent of the disease, is an enveloped RNA virus belonging to the genus *Morbillivirus* in the family *Paramyxoviridae*. MV has two envelope glycoproteins, the hemagglutinin (H) and fusion (F) proteins, both of which are necessary for MV entry and syncytium formation. The H protein has the ability to bind to a host cell receptor, whereas the F protein causes the fusion of the viral envelope with a cell membrane upon the H protein's binding to a receptor. MV infects immune cells by using human signaling lymphocyte activation molecule (SLAM; also called CD150) as a receptor (2). Vaccine strains of MV use CD46, which is expressed on all human cells except for red blood cells, as an alternate receptor (3, 4). Although wild-type (WT) MV cannot use CD46 as a receptor, pathological data from humans and experimentally infected monkeys showed that MV also infects SLAM-negative epithelial tissues in various organs, such as the skin, oral mucosa, pharynx, trachea, esophagus, intestines, and urinary bladder (5–12). In addition, WT MV induces large syncytia in primary human small airway epithelial cells (13). Recent studies showed that MV infects polarized epithelial cell lines in a SLAM- and CD46-independent manner (14, 15). Thus, there must be another receptor for MV, which is different from SLAM and CD46. However, this epithelial cell receptor for MV has not been identified. MV buds from the apical surface of polarized epithelial cells (15, 16). Furthermore, Leonard *et al.* (16) reported that a recombinant MV that cannot use the epithelial cell receptor still infects rhesus monkeys and causes a rash and anorexia after intranasal infection but is not shed in the airways. These data suggested that infection of polarized epithelial cells is important for the spread of MV to a new host.

Epithelial-mesenchymal transition (EMT) is a process by which epithelial cells lose their cell junctions and acquire a mesenchymal cell-like phenotype (17). EMT is observed in some developmental processes as well as cancer invasion and metastasis. Snail, a transcriptional repressor, plays a central role during EMT (17, 18). Although the functions of Snail remain to be elucidated, some targets of Snail have been reported. For example, Snail inhibits expression of the gene encoding E-cadherin, which constitutes the adherens junction (19, 20). Snail also acts as a transcriptional repressor of genes encoding tight junction-related molecules, such as occludin, claudins, and Crumbs3 (21–24), and of other genes (25, 26). Furthermore, Ohkubo *et al.* reported that Snail down-regulates claudin-1 at the post-transcriptional level (22).

In this study, we show that a lung adenocarcinoma cell line II-18 (27), which forms tight junctions, is highly susceptible to WT MV. When Snail was transiently expressed in II-18 cells, they lost adherens and tight junctions. Microarray analysis also confirmed the induction of EMT in II-18 cells. Importantly,

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[2] The abbreviations used are: MV, measles virus; WT, wild-type; EMT, epithelial-mesenchymal transition; GFP, green fluorescent protein; EGFP, enhanced green fluorescent protein; MOI, multiplicity of infection; p.i., postinfection; H protein, hemagglutinin protein; F protein, fusion protein; SLAM, signaling lymphocyte activation molecule; hSLAM, human SLAM; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; mAb, monoclonal antibody; CIU, Cell infectious units; Z-, benzyloxycarbonyl; CHO, Chinese hamster ovary.