ORIGINAL ARTICLE

Orphan nuclear receptor HNF4G promotes bladder cancer growth and invasion through the regulation of the *hyaluronan synthase 2* geneT Okegawa¹, K Ushio², M Imai², M Morimoto³ and T Hara³

Nuclear receptors (NRs) are a class of transcription factors that are closely involved in the progression of certain types of cancer. We aimed to study the relation between bladder cancer and NRs, with special focus on orphan NRs whose ligands and functions have not been identified. First, we examined the expression levels of 22 genes encoding orphan NRs in clinical bladder cancer and found that hepatocyte nuclear factor 4 γ (*HNF4G*; *NR2A2*) and *NR2F6* were the genes that were upregulated most frequently in cancer tissues compared with their paired normal tissues. Knockdown and overexpression of each of these orphan NRs suppressed and stimulated the growth of bladder cancer cells *in vitro*, respectively. HNF4G also promoted tumor growth in bladder cancer xenograft models *in vivo*. Furthermore, HNF4G was both necessary and sufficient for the invasion of bladder cancer cells *in vitro*. Moreover, using microarray analyses, we identified *hyaluronan synthase 2* (*HAS2*) as one of the genes induced by HNF4G in bladder cancer cells. Transcription was activated by HNF4G in reporter assays using the promoter/enhancer region of the *HAS2* gene. The endogenous expression of the *HAS2* gene was suppressed by knockdown of HNF4G. In turn, knockdown of *HAS2* inhibited the growth and invasion of bladder cancer cells. Taken together, our data suggest that some orphan NRs are involved in bladder cancer progression and that, among them, HNF4G promotes the growth and invasion of bladder cancer, at least in part, via the regulation of the *HAS2* gene.

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INTRODUCTION

Nuclear receptors (NRs) are a class of transcription factors that regulate gene expression in a spatiotemporal manner, thereby controlling differentiation, homeostasis and metabolism. Forty-eight NRs are known in humans.¹ Twenty-five of them are referred to as orphan NRs and their endogenous ligands and biological functions have not been identified.²

Bladder cancer can be categorized into two major clinicopathologically different tumor subtypes: superficial, non-muscle-invasive type and advanced, muscle-invasive type.³ Patients with superficial bladder cancer are usually treated with transurethral resection with or without intravesical chemotherapy and have a favorable prognosis; however, some of these patients suffer from recurrence with grade progression. Patients with advanced bladder cancer are treated with more aggressive therapeutic options such as radical cystectomy and urinary diversion with or without chemotherapy; however, these patients have a less favorable prognosis with a 5-year survival rate of approximately 50% in the United States of America.⁴ Clinicopathological parameters such as grade, stage and invasion provide important prognostic information but are not sufficient for the precise prediction of recurrence or survival in patients with bladder cancer. Therefore, novel biomarkers that predict and therapeutic options that prevent the progression of this disease need to be developed.

Recently, orphan NRs such as Nur77 (NR4A1) and Nurr1 (NR4A2) were reported as being involved in bladder cancer progression.^{5,6}

Moreover, the relation between orphan NRs and cancer progression—for example, ERR α (ESRRA; NR3B1) and LRH-1 (NR5A2) in breast cancer;^{7,8} EAR-2 (NR2F6), Nurr1 and ROR α (RORA; NR1F1) in colorectal cancer;^{6,9,10} and DAX1 (NR0B1) in lung cancer¹¹—have been reported in other types of cancer. However, to our knowledge, no comprehensive expression analyses of orphan NRs have been performed using paired clinical cancer and normal tissues, and the relation between orphan NRs and cancer progression remains unclear.

In this study, we investigated the relationship between orphan NRs and bladder cancer progression. First, we studied the expression of 22 orphan NRs in paired clinical bladder cancer tissues and adjacent normal tissues from the same patients. Subsequently, we selected the hepatocyte nuclear factor 4 γ (HNF4G; NR2A2) and NR2F6 for further analysis to study the involvement of these orphan NRs in the growth and invasion of bladder cancer cells.

RESULTS

Orphan NR expression in clinical bladder cancer tissues

First, we studied the expression of 22 orphan NRs in human bladder cancer tissues and in their paired adjacent normal bladder tissues. Thirty-two pairs of samples were tested. Upregulation of *HNF4G* and *NR2F6* in cancer tissues was observed in 19 of 32 (59%, $P=0.0356$) and 16 of 32 (50%, $P=0.0030$) cases, respectively

¹Department of Urology, Kyorin University School of Medicine, Mitaka, Japan; ²Extra Value Generation and General Medicine Drug Discovery Unit, Takeda Pharmaceutical Company Limited, Fujisawa, Japan and ³Oncology Drug Discovery Unit, Takeda Pharmaceutical Company Limited, Fujisawa, Japan. Correspondence: Dr T Hara, Oncology Drug Discovery Unit, Takeda Pharmaceutical Company Limited, 26-1, Muraoka-Higashi 2-chome, Fujisawa, Kanagawa 251-8555, Japan.

E-mail: takahito.hara@takeda.com

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Table 1. Messenger RNA expression levels of orphan nuclear receptors in bladder cancer tissues

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		HNF4G	RORC	ESRRG	COUP-1TFII	EAR-2	TR2	Rev-Erb- β	GCNF	Rev-Erb- α	LRH-1	TR4	COUP-1TFI	PNR	NORI	ESRRA	NUR77	NURR1	FTZF1	TLX	HNF4A	ESRRB	RORA				
Patient No.	Stage	NR2A2	NR1F3	NR3B3	NR2F2	NR2F6	NR2C1	NR1D2	NR6A1	NR1D1	NRH-1	NR2C2	NR2F1	NR2E3	NR4A3	NR3B1	NR4A1	NR4A2	NR5A1	NR2E1	NR2A1	NR3B2	NR1F1				
1	G3	T2a	4.37	4.33	0.54	1.35	2.76	1.46	1.70	1.88	0.59	1.66	0.09		0.02	3.42	0.01	0.07									
2	G2	T3a		0.57	11.21	1.48	2.13	3.19	2.23		1.75	2.89	2.06		0.53	0.93	0.93	1.11									
3	G3	T3b	4.78	0.31	7.16	3.17	6.43	2.96	1.37	5.47	4.59	0.39	1.60	0.34	7.95	0.07	1.57	0.17	52.72			7.50					
4	G3	T2b	0.83	2.05	2.76	1.18	0.96	1.25	1.63	0.60	1.35	0.96	1.30	1.96	0.99	0.63	0.82	1.09	0.80								
5	G3	T1	5.09	0.39	0.97	2.55	1.34	1.12	1.56	1.67	1.27	1.17	0.97		0.20	0.63	0.21	0.12									
6	G3	T3b	4.30	3.06	0.98	1.55	1.07	0.76	0.71	1.98	0.60	1.33	0.84	1.08	1.72	0.89	1.00	0.62	0.87	0.76			1.26				
7	G3	T1	1.17	0.21	0.64	0.28	1.17	0.58	0.74	1.03	0.49	0.18	0.58	0.09	0.15	0.01	1.38	0.03	0.02		0.77						
8	G2	T1		1.42	1.00	1.13	0.81	1.15	1.04	1.24	0.85	1.22	0.79	0.61	1.17	0.32	0.59	0.59	0.68			0.46					
9	G3	T3a	0.53	0.13	0.82	0.13	0.60	0.39	0.70	1.18	0.37	0.23	0.35	0.13	0.10	0.29	1.42	0.06	0.13		2.20		0.41				
10	G3	T1		0.74	0.63	2.60	1.67	3.39	2.32	0.56	3.11	0.91	3.55	2.78	2.96	3.31	0.85	5.23	1.91				2.67				
11	G2	Ta	11.22	0.22	3.02	1.23	1.65	1.40	1.58	6.28	0.67	0.67	1.06	0.40	0.98	0.07	1.81	0.10	0.33			0.13					
12	G3	T3b	2.70	0.23	7.61	0.85	0.64	1.42	1.69	0.91	0.34	0.27	1.01	0.33	0.30	0.05	1.10	0.02	0.10			2.17					
13	G3	T4	6.56	3.48	0.12	0.51	1.10	0.75	1.14	1.96	0.18	0.24	0.63	0.14	0.05	0.40	0.10	0.15				1.07					
14	G3	T1	1.89	1.82	1.38	5.70	3.03	1.80	2.55	0.26	1.12	1.13	1.80	2.52	1.84	0.49	1.38	1.35	0.65								
15	G3	T2a	0.79	0.03	0.10	0.27	1.91	2.30	4.09	0.18	0.42	0.33	1.20	1.04	0.36	0.01	0.33	0.02	0.01				0.38				
16	G3	T2a	3.30	3.54	22.58	17.11	12.61	9.21	12.97	7.61	20.02	1.52	3.50	9.44	4.86		1.92	0.75									
17	G2	T2a	0.31	0.24	0.26	0.19	0.50	0.22	0.23	0.43	0.28	0.13	0.24	0.10	0.38	0.01	1.18	0.00	0.01	0.55		0.15					
18	G3	T2a	0.33	0.21	0.02	0.19	1.00	0.16	0.36	1.40	0.10	0.18	0.23	0.19	0.13	0.05	1.09	0.05	0.14		1.05	0.41					
19	G3	T2b	4.03	5.42	0.23	0.86	4.55	1.40	6.10	3.07	0.72	0.21	1.69	0.16	0.31	0.05	1.09	0.03	0.07								
20	G3	T4		2.57	6.28	1.61	1.07	2.75	1.99		1.72	7.66	2.15	0.53	3.69	0.45	3.27	2.29		11.13	9.89						
21	G3	T2b	1.59	1.32	0.02	1.92	3.39	0.24	0.35		0.31	0.06	0.42	0.09	0.17	0.06	1.41	0.08	0.08								
22	G3	T1s	7.90	2.14	10.89	4.60	2.85	4.57	2.69		2.84	13.90	5.88	3.52	11.28	1.49	4.87	4.71									
23	G3	T2a	2.66	0.46	0.20	0.61	0.55	0.59	0.58	0.34	0.32	0.52	0.65	0.34	0.19	0.22	0.59	0.12	0.14			0.25					
24	G3	T1	69.64	7.58	0.59	3.19	1.45	1.68	1.10	2.44	2.16	4.17	1.97	4.17	3.08	2.45	1.46	2.64	4.96								
25	G3	T2a	30.25	6.29	0.65	2.33	2.26	1.67	0.97	1.10	0.72	1.97	1.87	2.71	0.63	1.25	0.85	1.54	1.39								
26	G2	T4	0.01	0.72	0.05	0.50	0.80	0.30	0.29	0.59	0.10	0.05	0.20	0.08	0.16	0.02	0.31	0.04	0.03	17.02	1.30						
27	G3	T2a	1.97	1.04	1.60	0.49	1.33	0.98	1.09	0.94	1.49	0.49	0.67	0.08	0.06	0.23	1.11	0.26	0.99	3.30	7.21						
28	G3	T1	0.34	1.38	0.17	1.46	2.53	1.05	0.61		0.45	1.78	1.10	2.41	4.14	0.48	1.00	0.39	0.57								
29	G3	T2b	18.30	5.94	0.09	0.68	3.50	0.99	1.00	4.17	2.88	0.24	1.28	0.10	0.03	0.74	0.74	0.02	0.05			0.31					
30	G3	T1	26.80	6.50		15.26	5.88	11.19	5.46		8.33	9.52	9.75	3.85	3.40	1.97	4.46	3.14									
31	G3	T3b	2.61	1.97	0.00	0.11	0.99	0.31	0.13	1.52	0.06	0.11	0.01	0.12		0.01	0.46	0.01	0.03								
32	G3	T4	1.76	6.26	0.20	5.13	2.97	2.49	1.82	0.49	2.12	0.85	2.05	5.07	4.65	0.47		0.77	0.50								

The figure in the column expresses the ratio of mRNA expression levels of bladder cancer tissues relative to normal bladder tissues. Columns colored red indicate increase in cancer tissues (fold change > 1.5), those colored green indicate decrease in cancer tissues (fold change < 0.5), and those columns marked ND indicate the expression could not be detected in both cancer and normal tissues. * $p < 0.05$, ** $p < 0.01$ by Welch's t -test.

(Table 1). The other orphan NRs that were upregulated in cancer tissues were as follows: *NR1D2* in 15 out of 32 (47%, $P=0.0333$), *RORC* in 15 of 32 (47%, $P=0.0346$), *ESRRG* in 10 of 32 (31%, $P=0.0443$), *NR2F2* in 13 of 32 (41%, $P=0.0354$), *NR2C1* in 12 of 32 (38%, $P=0.0266$), *NR2C2* in 13 of 32 (41%, $P=0.0482$) and *NR6A1* in 10 of 25 (40%, $P=0.0309$) cases (Table 1). Conversely, down-regulation of *NR4A1*, *NR4A2* and *NR4A3* in cancer tissues was observed in 19 (59%, $P=0.9079$), 18 (56%, $P=0.5021$) and 22 (69%, $P=0.7842$) cases, respectively, and the expression levels of *RORA*, *HNF4A* (*NR2A1*), *TLX* (*NR2E1*), *ESRRB* (*NR3B2*) and *NR5A1* were below the detection limit in both cancer and normal tissues (Table 1).

HNF4G and NR2F6 promote the growth of bladder cancer cells *in vitro*

Because HNF4G and NR2F6 were among the top orphan NRs that were upregulated most frequently in bladder cancer tissues, and HNF4G and NR2F6 have been reported to be involved in cancer progression,^{9,12–14} we selected HNF4G and NR2F6 for further analysis using bladder cancer cell lines. To study the involvement of these orphan NRs in the growth of bladder cancer cells, first we prepared RT-4 and UM-UC-3 cells overexpressing HNF4G. The growth rate of the RT-4 and UM-UC-3 cells overexpressing HNF4G was significantly higher than that of the control cells overexpressing LacZ (Figure 1a). In addition, small interfering RNAs (siRNAs) against *HNF4G* significantly suppressed the growth of T24

bladder cancer cells (Figure 1b), demonstrating that HNF4G is both necessary and sufficient for the growth of bladder cancer cells. Similar results were obtained for NR2F6 (Figures 1a, b). We also confirmed that HNF4G and NR2F6 were both necessary and sufficient for the growth of A549 lung cancer cells (Supplementary Figure S1), and the cells treated with siRNA against *HNF4G* and *NR2F6* were arrested in the G1 phase with a decrease in the proportion of cells in the S phases of the cell cycle (Supplementary Figure S2).

HNF4G promotes bladder tumor growth *in vivo*

To study whether HNF4G and NR2F6 also promote tumor growth *in vivo*, we injected the RT-4 cells overexpressing these orphan NRs subcutaneously into nude mice. Overexpression of HNF4G significantly accelerated tumor growth compared with overexpression of the LacZ control (Figure 2a). However, overexpression of NR2F6 did not accelerate RT-4 tumor growth (Figure 2a). The same tendency was observed in tumors formed by UM-UC-3 cells overexpressing HNF4G or NR2F6 (Figure 2b).

HNF4G promotes invasion of bladder cancer cells

The invasive and growth-promoting properties of cancer cells have critical roles in cancer progression. The invasive ability of the UM-UC-3 cells overexpressing HNF4G was significantly higher than that of the control cells overexpressing LacZ (Figure 3a). Similar results were obtained for NR2F6 (Figure 3a). In contrast, siRNAs

Figure 1. Hepatocyte nuclear factor 4 γ (HNF4G) and NR2F6 are necessary and sufficient for proliferation in bladder cancer cells. **(a)** Growth assay and western blot of HNF4G and NR2F6 in RT-4 and UM-UC-3 cells; the number of cells was counted on day 8 (RT-4) or day 3 (UM-UC-3) after seeding LacZ-, HNF4G- or NR2F6-overexpressing RT-4 (4×10^4) and UM-UC-3 (2×10^4) cells. Horse serum instead of fetal bovine serum was used for RT-4 cells. Data are shown as means $\pm$ s.d. $n=4$, * $P<0.05$, ** $P<0.01$ by parametric Dunnett's test. **(b)** Growth assay, mRNA quantification and western blot of HNF4G and NR2F6 in T24 cells; the number of cells was counted on day 3 after transfection with control small interfering RNA (siRNA) or siRNA against NR2F6 or HNF4G in T24 cells. Data are shown as means $\pm$ s.d. $n=4$, ** $P<0.01$ by parametric Dunnett's test.

Figure 2. Overexpression of hepatocyte nuclear factor 4 γ (HNF4G) promotes tumor growth *in vivo*. (a) LacZ-, HNF4G- or NR2F6-overexpressing RT-4 cells (3×10^5 cells per mouse, right flank) were inoculated into nude mice, and the tumor volume was measured once a week. Means $\pm$ s.d. $n = 10$. * $P < 0.05$, ** $P < 0.01$ by parametric Dunnett's test. (b) LacZ-, HNF4G- or NR2F6-overexpressing UM-UC-3 cells (3×10^5 cells per mouse, right flank) were inoculated into nude mice, and the tumor volume was measured twice a week. Means $\pm$ s.d. $n = 10$. ** $P < 0.01$ by parametric Dunnett's test.

Figure 3. Hepatocyte nuclear factor 4 γ (HNF4G) regulates cell invasion in bladder cancer cells. (a) The effect of overexpression of HNF4G or NR2F6 on the invasive properties was examined in UM-UC-3 cells using the Collagen IV-coated Boyden chamber. The invasion rates of control cells are normalized to 100% and those of cells overexpressing orphan NRs are expressed as percentage compared with control. Data are shown as means $\pm$ s.d. $n = 6$, * $P < 0.05$, ** $P < 0.01$ by parametric Dunnett's test. (b) The effect of knockdown of HNF4G or NR2F6 on the invasive properties was examined in T24 cells using the Collagen IV-coated Boyden chamber. The invasion rates of the control cells are normalized to 100% and those of cells treated with small interfering RNA (siRNA) against orphan NRs are expressed as percentage compared with control. Data are shown as means $\pm$ s.d. $n = 6$, * $P < 0.05$, ** $P < 0.01$ by parametric Dunnett's test.

against *HNF4G*, but not those against *NR2F6*, significantly suppressed invasion in T24 bladder cancer cells (Figure 3b), indicating that only HNF4G is both necessary and sufficient for the invasion of bladder cancer cells. The suppression of invasion by *HNF4G* siRNA was not due to inhibition of cell attachment but due to inhibition of cell motility (Supplementary Figure S3). We also confirmed that HNF4G was both necessary and sufficient for the invasion of A549 lung cancer cells (Supplementary Figure S4).

Identification of the *HNF4G* downstream genes responsible for growth and invasion in bladder cancer cells

Because HNF4G, but not NR2F6, promoted tumor growth *in vivo* and was both necessary and sufficient for invasion *in vitro*, we selected HNF4G for further analysis. To study the molecular mechanisms underlying the HNF4G-mediated promotion of both the growth and invasion of bladder cancer cells, we used a microarray analysis to compare the gene expression profile of the RT-4 cells overexpressing HNF4G with that of cells overexpressing LacZ. We detected 53 and 71 genes that were upregulated and downregulated, respectively, in the cells overexpressing HNF4G (Supplementary Table S1). Among the 53 upregulated genes, 11 genes related to cancer or cell proliferation, including *REG4*, *HAS2*, *AREG* and *PTGS2*, were confirmed as being upregulated in the cells overexpressing HNF4G using quantitative PCR (Figure 4a). Among these four genes, the expression of the *HAS2* gene exclusively was suppressed in T24 cells by an siRNA against *HNF4G* (Figure 4b), suggesting that *HAS2* gene expression is tightly regulated by

HNF4G. The promoter/enhancer region of the *HAS2* gene includes HNF4-responsive sequences. Reporter assays revealed that HNF4G transactivated the transcription via the *HAS2* promoter/enhancer region (Figure 4c). Finally, to determine whether *HAS2* expression is associated with cell growth and invasion, we examined the effect of an siRNA against *HAS2* in bladder cancer cells. *HAS2* knockdown suppressed both the growth and invasion of T24 cells (Figures 4d, e), suggesting that HNF4G has a role in the cell growth and invasion of bladder cancer cells at least in part via the regulation of the expression of the *HAS2* gene.

DISCUSSION

In this study, we investigated the relationship between orphan NRs and bladder cancer progression. First, we studied the expression of 22 orphan NRs in paired clinical bladder cancer tissues and adjacent normal tissues from the same patients. To our knowledge, this is the first study that examined the expression of orphan NRs comprehensively using paired clinical tissues.

HNF4G and *NR2F6* were among the top orphan NRs that were upregulated most frequently in bladder cancer tissues. On the basis of these clinical findings, we hypothesized that orphan NRs such as HNF4G and NR2F6 have a critical role in bladder cancer progression. Regarding the relation between upregulation and clinical features, higher expression of *HNF4G* showed a tendency to be correlated with higher grade. Among the five grade 2 (low grade) tumors, four did not express or upregulate

Figure 4. Hyaluronan synthase 2 (HAS2) messenger RNA (mRNA) expression levels are regulated by hepatocyte nuclear factor 4 γ (HNF4G), and knockdown of HAS2 suppresses cell proliferation in bladder cancer cells. **(a)** REG4, HAS2, AREG and PTGS2 mRNA expression levels were determined by real-time PCR. The expression levels in the RT-4 cells overexpressing HNF4G are expressed as ratio by setting the expression levels in the control LacZ-overexpressing cells as 1, $n=2$. **(b)** HAS2 mRNA expression was determined by quantitative RT-PCR at 72 h after transfection with the control small interfering RNA (siRNA) or siRNA against HNF4G in T24 cells. Data are shown as means $\pm$ s.d. $n=4$, $^{*}P<0.01$ by parametric Dunnett's test. **(c)** Cells were seeded in a 96-well plate at 24 h after transfection with mock/pCMV or HNF4G/pCMV, together with TK/pGL4.15 or HAS2-1000/pGL4.15 in UM-UC-3 cells. TK/pGL4.15 luciferase activity was used as an internal transfection control. Data are shown as means $\pm$ s.d. $n=3$, $^{**}P<0.01$ by Student's t -test. **(d)** Growth assay, mRNA quantification and western blot of HAS2 in T24 cells; the number of cells was counted 72 h after transfection with the control siRNA or siRNA against HAS2 in T24 cells. Data are shown as means $\pm$ s.d. $n=3$, $^{*}P<0.05$, $^{**}P<0.01$ by parametric Dunnett's test. **(e)** The invasion rates of the control cells are normalized to 100% and those of cells treated with siRNA against HAS2 in T24 cells are expressed as percentage compared with control. Data are shown as means $\pm$ s.d. $n=4$, $^{**}P<0.01$ by parametric Dunnett's test.

HNF4G. However, because of the very limited sample size, further study is required to clarify the relation.

HNF4G is one of the two isoforms of human HNF4 (the other isoform being HNF4 α). Much progress has been made in understanding the function of HNF4 α , such as its involvement in liver development and metabolism¹⁵ and its possible role in cancer.^{16,17} In contrast, very few studies have focused on HNF4G. Among these reports, it was shown that the expression levels of HNF4G were increased in five of six clinical human hepatocellular carcinoma samples,¹² and this molecule was highlighted as a susceptibility factor for pancreatic cancer.¹³ However, the role of HNF4G in cancer remains completely unclear. NR2F6, also known as EAR-2, has been reported to be involved in cell growth and differentiation in leukemia.¹⁴ In addition, recently it was reported to correlate with the progression of colorectal cancer via X-linked inhibitor of apoptosis protein (XIAP)-mediated antiapoptotic signaling.⁹

Regarding the expression of orphan NRs in clinical bladder cancer, Inamoto *et al.*⁶ showed that the expression of Nurr1 in the

cytoplasm correlates with adverse outcomes in patients with bladder cancer. It has also been reported that Nur77 is overexpressed in clinical bladder cancer tissues compared with nontumor bladder tissues.⁵ In contrast, our data did not demonstrate the upregulation of Nur77 or Nurr1; these two orphan NRs were downregulated in more than half of the patients examined in this study. Because these authors detected the Nur77 and Nurr1 proteins using immunohistochemical staining, the discrepancy between their results and our data may be due to the differences in the detection methods used. Inamoto *et al.* detected the cytoplasmic mislocalization.

Our *in vitro* study showed that HNF4G was both necessary and sufficient for the growth of bladder cancer cells. In addition, HNF4G also promoted tumor growth in bladder cancer xenograft models *in vivo*, supporting the hypothesis that these two orphan NRs have a critical role in bladder cancer progression. NR2F6 was also necessary and sufficient for the growth of bladder cancer cells *in vitro*, which is consistent with findings described for leukemia.¹⁴ However, NR2F6 did not promote tumor growth in our *in vivo*

study, for unknown reasons. As a tentative explanation, the nature of the involvement of NR2F6 in angiogenesis or cancer stroma interaction may be different from that of HNF4G; alternatively, the experimental conditions used in this study may not have been suitable for the growth of NR2F6-expressing cancer cells.

Notably, HNF4G was also necessary and sufficient for the invasion of bladder cancer cells. This finding further confirmed the hypothesis that HNF4G has a critical role in bladder cancer progression. With respect to the link between invasion and NRs, we previously reported that the androgen receptor promotes prostate cancer invasion.¹⁸ Recently, it was shown that the orphan NR LRH-1 promotes, whereas ROR α suppresses, breast cancer invasion.^{8,10} These findings suggest that NRs may regulate cancer progression by modulating the expression of genes related to both growth and invasion.

On the basis of the findings that HNF4G was upregulated in clinical bladder cancer tissues and that HNF4G had a critical role in both growth and invasion in experimental bladder cancer models, we selected HNF4G as a potential drug target or biomarker and examined further the mechanism of action of HNF4G in bladder cancer growth and invasion. Because NRs are a class of transcription factors, we studied the genes whose expression was induced by overexpression of HNF4G in bladder cancer cells. Many genes were induced by HNF4G, including the *HAS2* gene. Our subsequent molecular experiments demonstrated the close relation between HNF4G and *HAS2*. *HAS2* is one of the three HAS isoforms and catalyzes hyaluronic acid (HA) synthesis.¹⁹ Kramer *et al.*²⁰ reported that the expression of members of the HA family, such as *HYAL1* and *HAS1*, predicted bladder cancer metastasis and that the combined expression of *HAS2* and *HYAL1* predicted its recurrence significantly. Consistently, *HAS1* regulates bladder cancer growth, invasion and angiogenesis.²¹ *HAS2* and *HAS3* have also been shown to promote tumor growth and metastasis.^{22–24} HA is a nonsulfated glycosaminoglycan that is involved in many physiological functions, including tumorigenesis.²⁵ Moreover, the elevation of the levels of HA in urine is reportedly a diagnostic marker of bladder cancer.^{26–28} Taken together, HNF4G might promote bladder tumor progression through creation of an HA-rich environment by regulating the *HAS2* gene.

In this study, approximately 70% of patients (23/32) exhibited upregulation of at least one of *HNF4G* and *NR2F6*. These orphan NRs may work in a concerted manner to mediate bladder cancer progression. Furthermore, hOGG1 (OGG1), N-cadherin (CDH2) and TP53 have been identified as candidate biomarkers of bladder cancer,^{29–33} although further studies are necessary to clarify their usefulness in a clinical setting. Orphan NRs may be involved in bladder cancer progression through regulation of the expression of these candidate molecules or by functioning in a concerted manner with them.

Androgen receptor and estrogen receptor (ESR1) are central growth signals in prostate and breast cancers, respectively. Inhibition of the signaling of these NRs is the main form of medical therapy for these types of cancers.^{34–37} Moreover, there is increasing evidence that NRs, such as the androgen receptor and estrogen receptor, are associated with the development and progression of bladder cancer,³⁸ although this contention remains controversial and the therapies used to target the androgen receptor or estrogen receptor have not been applied to bladder cancer patients. In this study, we demonstrated that orphan NRs are also possible therapeutic targets for bladder cancer. Our data also suggest that the importance of orphan NRs may not be limited in bladder cancer but may expand to other types of cancer.

In conclusion, our results suggest that (1) some orphan NRs, such as HNF4G and NR2F6, are involved in bladder cancer progression; (2) among them, HNF4G promotes both the growth and invasion of bladder cancer cells; (3) *HAS2* is one of the *HNF4G* downstream genes that is responsible for the growth and invasion

of bladder cancer; and (4) orphan NRs such as HNF4G are possible novel therapeutic targets and biomarkers for bladder cancer.

MATERIALS AND METHODS

Reagents

The primers/probes used in this study were purchased from Applied Biotechnology (Carlsbad, CA, USA) (Supplementary Tables S2 and S3).

Clinical samples

Clinical bladder cancer tissues and normal tissues adjacent to them were obtained from 32 patients who received radical cystectomy at the Kyorin University Hospital (Mitaka, Tokyo, Japan). Our institutional committees approved the experiments, and informed consent was obtained from all patients. The clinical and pathological features of the patients are shown in Table 2. The tissues obtained were soaked in liquid nitrogen until the analyses.

Cell lines

The human bladder cancer T24, RT-4, UM-UC-3 and HEK293T cell lines were purchased from the American Type Culture Collection (ATCC) and used within 6 months after receipt; authentication of cell lines was performed by ATCC. T24 and RT-4 cells were cultured in McCoy's 5A medium containing 10% fetal bovine serum. UM-UC-3 and HEK293T cells were cultured in high-glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum.

Cell proliferation assay

Cells were plated in 24-well plates at a density of 2×10^4 cells per well unless otherwise noted in the figure legends. After 3–8 days of incubation at 37 °C in 5% CO₂, the cells were trypsinized and counted using a particle counter (Beckman Coulter, Fullerton, CA, USA). Knockdown by siRNA was performed using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) via the reverse transfection method and according to the manufacturer's protocol.

Quantitative real-time PCR

Total RNA was isolated from bladder tissues and cells using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Single-stranded cDNAs were synthesized using Taqman Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. All assay plates were run on an Applied Biosystems 7900HT Fast Real-Time PCR System using standard settings (cycling program included a 10 min incubation at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min). The expression of the 18S ribosomal ribonucleic acid was used as an internal control.

Plasmid constructs

The full-length *NR2F6* (NM_005234) and *HNF4G* (NM_004133) genes were cloned from the MTC Panel I, small intestine (Clontech, Palo Alto, CA, USA). The cDNAs were inserted into the pLenti6.3/V5-DEST vector. To prepare the *HAS2* promoter luciferase (Luc) reporter, the total 1030 bp of the human *HAS2* promoter sequence was amplified by PCR from human

Table 2. Clinical and pathologic features for bladder cancer patients

No. of patients	32		
Mean age	69 (58–84)		
Sex (males/females)	27/5		
Stage	Grade 1, n	Grade 2, n	Grade 3, n
T1	0	2	8
T2a	0	1	7
T2b	0	0	4
T3a	0	1	1
T3b	0	0	4
T4	0	1	3

genomic DNA (Takara Bio, Shiga, Japan) using Pfx DNA polymerase (Invitrogen) and placed into the pGL4.15 plasmid (Promega, Madison, WI, USA).

Generation of cell lines stably expressing NRs using lentiviral particles

To obtain lentiviral particles to induce the expression of LacZ, NR2F6 or HNF4G, HEK293T cells (4.79×10^5 cells/ml) were seeded into a T-225 flask (50 ml, BD Bioscience, San Jose, CA, USA). After 24 h, cells were transfected with the pCAG-HIVgp and pCMV-VSV-G-RSV-Rev vectors, together with the LacZ/pLenti6.3, NR2F6/pLenti6.3 or HNF4G/pLenti6.3 vectors, using Lipofectamine 2000 according to the manufacturer's instructions. After 24 h, the medium was replaced. The medium containing lentiviral particles was collected 48 h later, filtered through 0.45 μ m filters, and ultracentrifuged at 4 °C at 100 000 *g* for 2 h on an OptiMAX apparatus (with MLA55, Beckman Coulter). The precipitate was suspended in Hank's balanced salt solution. Lentiviral titers were determined using an HIV-1 P24 Antigen ELISA kit (Bio Academia, Osaka, Japan). Infected cells were selected in the presence of 20 μ g/ml of blasticidin (Invitrogen).

Animal experiments

The protocols used in animal experiments were approved by the Takeda Experimental Animal Use and Care committee in accordance with NIH standards. Male/female BALB/cA Jcl-nu/nu mice (22–24 g, 7 weeks old) were used in the present study. The animals were housed under controlled environmental conditions (12 h light:12 h dark cycle) and acclimatized for at least 1 week. Food and tap water were available *ad libitum*. The animals were divided randomly into several groups of 10 animals each. Briefly, 3×10^5 cells suspended in 100 μ l of a mixture of medium/Matrigel (1:1) were injected into the right flank region of the animals. Tumor size was determined every 4 days using calipers and the formula $V = ab^2/2$, where *a* is the long axis and *b* is the short axis of the tumor.

Invasion and migration assays

A Cultrex 96-well Collagen IV Cell Invasion Assay kit (Trevigen, Gaithersburg, MD, USA), a 24-well BD BioCoat Tumor Invasion System (BD Bioscience) and a Cultrex 96-well Collagen IV Cell Migration Assay kit (Trevigen) were used according to the manufacturer's instructions. Twenty-four hours after seeding, the cells were stained with calcein AM (5 μ g/ml, Trevigen) for 60 min at 37 °C. Subsequently, fluorescence was measured in a Fluoroskan Ascent plate reader (Thermo Fisher Scientific, Waltham, MA, USA) and ARVO MX 1420 multilabel counter (PerkinElmer, Norwalk, CT, USA).

Microarray analysis

RNA labeling and hybridization were performed according to the protocol for one-color microarray-based gene expression analysis using a Quick Amp Labeling kit (Agilent Technologies, Palo Alto, CA, USA). The labeled cRNAs were scanned on an Agilent DNA Microarray Scanner using Scan Control software (Agilent Technologies). The gene expression data were analyzed using GeneSpring GX software (Agilent Technologies).

Reporter gene assays

The HAS2-1000/pGL4.15 or TK/pGL4.15 plasmids were transfected with pCMV (mock) or HNF4G/pCMV plasmids in UM-UC-3 cells. After 24 h, cells were collected using Trypsin-EDTA (0.25% Trypsin, 1 mM EDTA · 4Na; Invitrogen), and 1.2×10^4 cells per well were seeded into a 96-well plate (BD BioCoat, poly-D-lysine, 96-well white opaque, BD Bioscience). After 24 h, whole-cell extracts were added to 100 μ l of Bright-Glo reagent (Bright-Glo Luciferase Assay System, Promega). The fluorescence of the samples was measured on an ARVO X Light plate reader (PerkinElmer).

Statistical analyses

Data are presented as means $\pm$ s.d. (*in vitro* measurements) and as means $\pm$ s.e.m. (*in vivo* measurements). Statistical analyses were performed using the SAS PreClinical Package Ver. 5.0 software (SAS institute, Cary, NC, USA). To detect significant differences in messenger RNA levels between normal bladder tissues and bladder cancer tissues, the ratio of the mRNA levels of normal and cancer tissues in each patient was calculated and the Welch *t*-test was carried out using these values. Dunnett's test, Student's *t*-test or the Welch *t*-test was used to analyze growth and invasion assay data.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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