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Original Article

High expression of Rap2A is associated with poor prognosis of patients with hepatocellular carcinoma

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Abstract: Rap2A is overexpressed in a multitude of human cancers and plays an important role in cytoskeleton rearrangement, arteriogenesis and cell migration. However, its role and function in hepatocellular carcinoma (HCC) has not yet been explored. Here, we aimed to investigate the expression of Rap2A in HCC and the relationship between Rap2A and clinicopathologic features of patients. Western blot and quantitative real-time PCR (qRT-PCR) showed that Rap2A was remarkably upregulated in HCC tissues compared to adjacent normal liver tissues. Immunohistochemistry (IHC) showed that Rap2A was mainly localized in the cytoplasm rather than nuclei in HCC tissues. Overexpression of Rap2A was significantly correlated with tumor size (P=0.019), metastasis (P=0.002), pathological differentiation (P=0.028) and vascular invasion (P=0.017) in HCC. Kaplan-Meier analysis demonstrated that HCC patients with high Rap2A expression had shorter overall survival time than those with low Rap2A expression (P=0.011). Furthermore, High level of Rap2A was a risk factor for HCC patients according to Cox's proportional hazard regression (P=0.026). In summary, our results suggest that high expression of Rap2A is involved in HCC progression and might be a novel prognostic indicator for patients.

Keywords: Rap2A, hepatocellular carcinoma, prognosis, survival time

Introduction

Hepatocellular carcinoma (HCC) ranks sixth among malignant tumors in the world and is the third leading cause of cancer death [1-3]. Although there are numerous therapy methods available now, surgical resection for HCC is still the best choice for patients. Most HCC patients usually have advanced-stage cancer at the time of initial diagnosis and lose their chance for tumor resection. Even though HCC patients have undergone standardized treatment, they will still have a worse outcome with local recurrence and distant metastasis in a short time after surgical operation. Hence, it is extremely necessary to seek new molecular markers and invent efficient imaging technologies which can better diagnose patients with early-stage HCC and predict their survival time.

Rap2A is one member of the RAS oncogene family. The full-length cDNA of the Rap2A gene is located at 13q34 and contains an open reading frame of 549 bp, encoding 183 amino acids with a molecular mass of 20,434 [4]. Previous studies have shown that Rap2A plays a critical role in regulation of various cellular processes such as cytoskeletal reorganization, brush border formation and cell migration [5-7]. It has been reported that Rap2A is upregulated in several types of human cancers, including prostate cancer, follicular thyroid cancer, nasopharyngeal carcinoma [8-10]. However, no reports relate to Rap2A expression in HCC except one study which examined the high expression of Rap2A in human hepatoblastoma HepG2 cells [7].

Here, we speculated that Rap2A may take part in the progression and development of HCC. To verify our hypothesis, we adopted different approaches including western blot, qRT-RCR and immunohistochemistry to detect Rap2A in

HCC and gathered patients' pathological information for statistical analysis. In the present study, we showed that Rap2A is obviously upregulated in HCC and maybe regarded as a novel biomarker for HCC patients' prognosis.

Materials and methods

Clinical samples collection and follow-up

Clinical samples were achieved from the Chronic Liver Disease Biological Sample Bank, Department of Hepatobiliary Surgery, Zhongshan Hospital Xiamen University. Detailed procedures are described in our previous study [11]. The clinical samples were obtained from patients with HCC who were treated at Zhongshan Hospital between 2011 and 2014. Specimen collection was performed after obtaining informed consent from each patient, and the study was approved by the Ethics Committee of the hospital. Paired tumor and nontumor (with more than 2 cm distance from the primary tumor's edge) tissue samples from the same patient were frozen quickly in liquid nitrogen postoperatively and stored at -80°C for western blot and qRT-PCR. None of the patients had received chemotherapy or radiotherapy before surgical operation resection. The median age of the patients included was 55 years. In addition, complete follow-up information of 82 patients was obtained by reviewing the patients' medical records.

Western blot

Tissue lysates or cell lysates were subjected to SDS-PAGE, and then proteins were transferred onto polyvinylidene fluoride membranes (Millipore, Germany). The membranes were blocked in Tris-buffered saline (TBS; pH 7.4) containing 5% non-fat milk and 0.1% Tween-20, incubated with the primary antibody against Rap2 (1:1000, 610215, BD Transduction Laboratories) and β-Actin (1:1000, AT0001, CMCTAG) overnight at 4°C, washed three times in TBS containing 0.1% Tween-20, and followed by the secondary antibody labeled with horseradish peroxidase (Jackson, the USA) for 1 h at room temperature. Antibody binding was visualized using enhanced chemiluminescence reagents (Advansta, the USA). Quantification of band densitometry was performed using ImageJ with normalization to the loading control β-Actin. Relative expression of Rap2A is calculated as Rap2A/β-Actin (Figure S1 and Table S1).

Quantitative real-time PCR

Total RNA was extracted from tissues or cell samples using the Trizol reagent (Ambion/Life Technologies, the USA) according to the manufacturer's instructions. Quantitative real-time PCR for Rap2A mRNA detection was performed as follows, with primers designed and synthesized by BGI-Tech (BGI, China): reverse transcription of 1 μg RNA was performed with the prime ScriptTM RT reagent kit with gDNA eraser (TaKaRa Biotechnology Inc, Japan) and qRT-PCR was performed using FastStart Universal SYBR Green Master ROX (Roche, Switzerland) in a total volume of 20 μl with LightCycler® 96 Real-Time PCR System (Roche, Switzerland). The sequences of the primer pairs were as follows: Rap2A forward, 5'-ACAATGGTGGACGAACTCTTT-3', reverse, 5'-CAGAACAGCATGGGTCATCT T-3'; β-Actin forward, 5'-ATAGCACAGCCTGGATAGCAACGTAC-3', reverse, 5'-CACCTTCTACAATGAGCTGCGTGTG-3'. A dissociation procedure was performed to generate a melting curve for confirmation of amplification specificity. β-Actin mRNA was quantified in parallel as the reference control. Relative gene expression was calculated using LightCycler® 96 Software. All experiments were replicated three times independently.

Haematoxylin and eosin (H&E) and immunohistochemistry staining

Tissues were fixed with 10% neutral formalin, embedded in paraffin, and 3-μm-thick sections were prepared by the pathology technologist. For H&E staining, sections were deparaffinized and hydrated with a gradient of alcohols. After soaking in phosphate-buffered saline (PBS), sections were stained with H&E. For IHC, sections were stained using the streptavidin-peroxidase method. In short, sections were deparaffinized, hydrated and soaked in 3% H_2O_2 for 10 min at room temperature, and then incubated with Rap2(12) monoclonal antibody (1:500, sc-136138; SANTA CRUZ) at 4°C overnight. Biotinylated secondary antibody and diaminobenzidine (DAB) were purchased from Maixin Biotechnology (Fuzhou, China). Evaluation of Rap2A staining in HCC tissue sections was performed with semi-quantitative IHC assessment method [12].

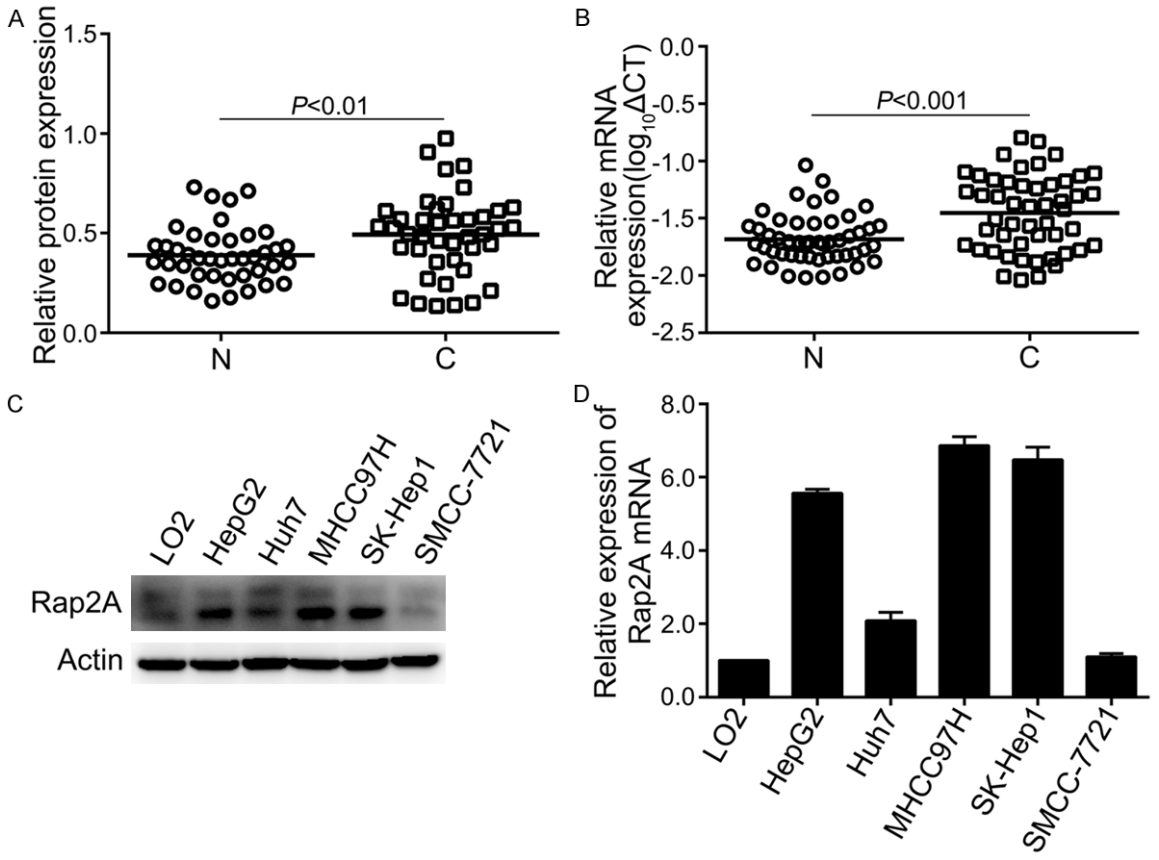

Figure 1. Upregulation of Rap2A protein and mRNA in HCC tissues and cell lines. A. Quantitative western blot analysis of Rap2A protein from 42 paired HCCs and their corresponding non-cancerous tissues. B. Analysis of Rap2A mRNA in 50 paired HCCs with their adjacent non-cancerous tissues by qRT-PCR method. Log10ΔCT of Rap2A mRNA were normalized to β-Actin mRNA. N, Non-cancer; C, Cancer. C, D. Rap2A protein and mRNA expression level in one normal hepatocytes (LO2), one hepatoblastoma cell line (HepG2) and 4 HCC cell lines (Huh7, MHCC97H, SK-Hep1, SMCC-7721).

Cell culture

The normal human hepatocytes cell line LO2, human hepatoblastoma cell line HepG2 and HCC cell lines including Huh7, MHCC97H, SK-Hep1 and SMCC-7721 were purchased from the cell bank of Shanghai Institute of Cell Biology. All cell lines were cultured in Dulbecco's modified Eagle's medium (HyClone, the USA) supplemented with 10% fetal bovine serum (Gibco, the USA) and 100 IU/ml penicillin and 100 μg/ml streptomycin (Millipore, Germany) at 37°C, 5% CO_2.

Statistical analysis

Data was analyzed using SPSS version 22.0 for Windows (IBM Corporation, New York, USA). The two-related samples Wilcoxon nonparametric test was performed to evaluate the difference of Rap2A expression between HCC tissues and adjacent normal liver tissues. The chi-squared test and Fisher's exact tests were used to examine possible correlations between Rap2A expression and clinicopathological features. Survival analysis was performed using the Kaplan-Meier test, and differences between curves were compared by the log-rank test. Multivariate analysis was performed to analyze the risk factors on postoperative survival. All *P* values were two-sided, and a *P* value of <0.05 was considered statistically significant.

Results

Rap2A is overexpressed in human HCC tissues and cell lines

Western blot for Rap2A in 42 paired HCC tissues and the quantification results are shown

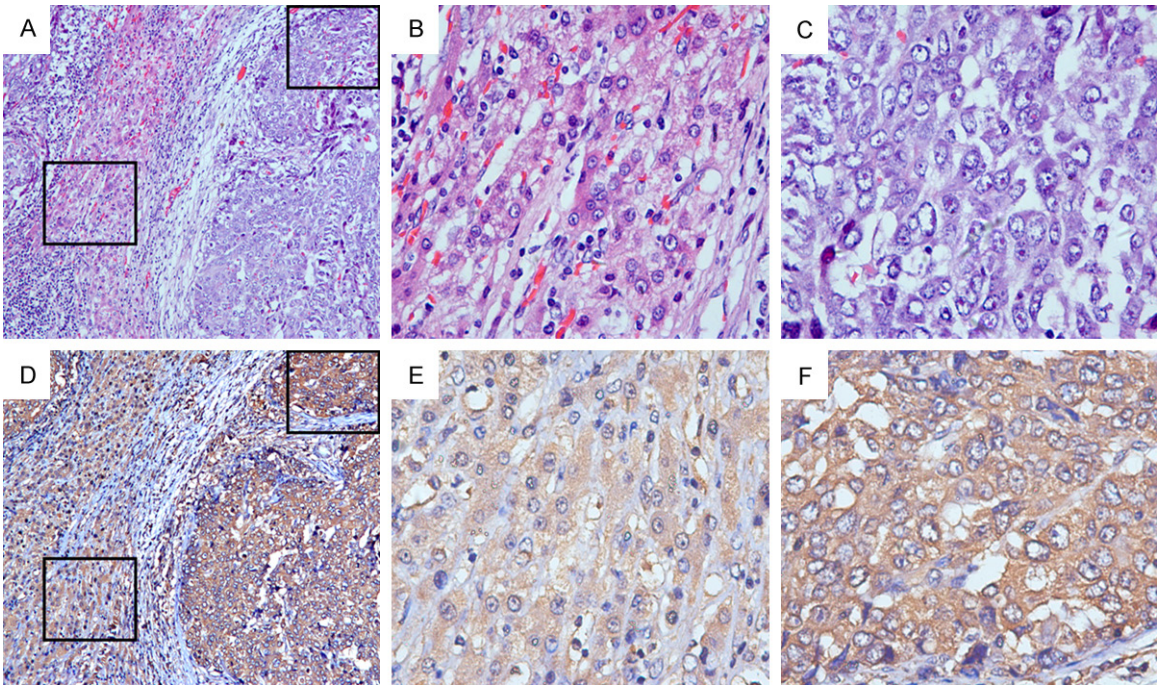

Figure 2. Representative immunostaining images of Rap2A in HCC. Stronger Rap2A expression was shown in HCC tissue (F) compared to relatively weak Rap2A expression in adjacent normal liver tissue (E). Rap2A was localized mainly in the cytoplasm and expressed little in the nuclei in HCC tissues (F). (A-C for H&E staining; D-F for IHC staining; Magnification: A, D 100×; B, C, E, F 400×).

in supplementary Figure S1 and Table S1. Rap2A protein was upregulated in HCC tissues (27 of 42=64.3%, $P<0.01$, **Figure 1A**). The mRNA expression of Rap2A was detected by qRT-PCR after extracting total RNA from 50 paired HCC tissues and neighboring non-cancerous tissues. As shown in **Figure 1B**, the expression level of Rap2A mRNA was higher in HCC than that in normal liver tissues (34 of 50=68.0%, $P<0.001$). We also found that Rap2A was overexpressed at protein and mRNA levels in Hep G2, MHCC97H and Sk-hep1 cells compared to weak expression in normal hepatocytes LO2 cells (**Figure 1C**, **1D**).

Immunohistochemical staining of Rap2A protein in HCC

IHC was adopted to identify the localization and expression pattern of Rap2A in 82 postoperative HCC samples. Rap2A was mainly detected in the cytoplasm and almost absent in the nuclei for HCC tissues (**Figure 2**). In addition, much stronger Rap2A staining was observed in HCC tissues (54 of 82=65.9%, $P<0.001$), while relatively weak Rap2A staining was examined in the majority of adjacent liver tissues.

Rap2A is correlated with HCC progression

After we detected Rap2A in 82 paired HCCs by IHC, we divided patients into a low Rap2A expression group (n=28) and a high Rap2A expression group (n=54). We then analyzed the clinical pathological correlation of Rap2A overexpression in HCC. High expression of Rap2A had strong correlation with tumor size ($P=0.019$), HCC metastasis ($P=0.002$), poor differentiation degree ($P=0.028$) and vascular invasion ($P=0.017$). On the contrary, there was no significant difference between high expression of Rap2A expression and patients' age, gender, liver cirrhosis status, serum alphafetoprotein (AFP) and hepatitis B virus (HBV) level (**Table 1**). Furthermore, we found that HCC patients with high expression of Rap2A had shorter survival time than patients with low expression of Rap2A ($P=0.011$) (**Figure 3**). We applied multivariate Cox proportional hazards regression analysis to explore the association between high Rap2A expression and HCC patients' prognosis. Our findings indicated that high expression of Rap2A was a risk factor related to the prognosis of patients with HCC (hazard ratio =3.090, with 95% confidence

Table 1. Correlation between Rap2A expression and clinicopathological features of HCC patients

	Variables	Rap2A expression level		χ^2	*P*
		Low (n=28)	High (n=54)		
Age (years)	<55	12	25	0.088	0.767
	≥55	16	29		
Gender	Female	6	9	0.280	0.597
	Male	22	45		
Tumor size	<5 cm	18	20	5.506	0.019*
	≥5 cm	10	34		
Metastasis	No	20	19	9.712	0.002*
	Yes	8	35		
Differentiation	Poor	4	22	7.129	0.028*
	Moderate	21	30		
	High	3	2		
Liver cirrhosis status	No	5	13	0.416	0.519
	Yes	23	41		
Serum HBV level (cps/ml)	<1000	15	28	0.022	0.882
	≥1000	13	26		
Serum AFP level (lg/l)	<400	19	37	0.004	0.951
	≥400	9	17		
Vascular invasion	No	17	18	5.651	0.017*
	Yes	11	36		

HBV, hepatitis B virus; AFP, Alpha-fetoprotein. *representative statistically significant (*P*<0.05).

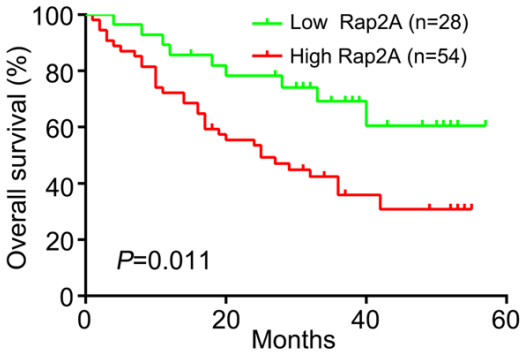

Figure 3. Kaplan-Meier analysis of overall survival between low and high Rap2A expression levels.

interval =1.142-8.362, *P*=0.026, **Table 2**). Meanwhile, patients' prognosis was associated with tumor size, metastasis status, tumor differentiation and vascular invasion (*P*<0.05, **Table 2**). Age, gender, liver cirrhosis status, serum AFP and HBV level showed no significant correlation with the prognosis of HCC.

Discussion

HCC is the most common type of liver cancer and the third leading cause of cancer death worldwide [13]. Exploring the molecular mechanism of tumorigenesis and recurrence is extremely important for seeking better therapeutic strategies for patients with HCC. The RAS oncogene family members can regulate a range of biological functions such as proliferation, migration and signal transduction in human cells [14, 15]. Rap2A belongs to the RAS oncogene family but its role and effect in hepatocellular carcinoma remain unknown.

We firstly examined the expression pattern of Rap2A in HCC tissues via western blot, qRT-PCR and immunohistochemistry. Rap2A was overexpressed in HCC tissues compared with neighboring nontumorous liver tissues. Rap2A was also upregulated in Hep G2, MHCC97H and Sk-hep1 cells at protein and mRNA levels. IHC showed that Pap2A was chiefly localized in the cytoplasm and significantly overexpressed in cancerous tissues. Then we analyzed the relationship between Rap2A expression and clinicopathological factors in HCC patients. Our results showed that high expression of Rap2A was of great clinical significance and correlated with tumor size, metastasis, differentiation and vascular invasion. The survival rate of high Rap2A expression group was much lower than that of low Rap2A expression group. Cox's proportional hazard regression also indicated that high Rap2A expression, tumor size, metastasis status, pathological differentiation and vascular invasion could predict for HCC patients' death.

Previous studies have proved that Rap2A can interact with and regulate several downstream effectors including MINK1, TNIK and MAP4K4 [5, 16]. Besides, High expression of nuclear p-TNIK may be useful for predicting the postoperative survival of HCC patients [17]. MAP4K4

Table 2. Cox regression model for prediction of 82 patients with HCC

Variable	Hazard ratio	95% CI	*P*-value
Tumor size	2.342	1.197-4.581	0.013*
Metastasis	2.166	1.019-4.605	0.045*
Differentiation	0.470	0.257-0.859	0.014*
Vascular invasion	2.994	1.375-6.517	0.006*
High Rap2A expression	3.090	1.142-8.362	0.026*

CI, Confidence interval; SE, standard error. *representative statistically significant ($P<0.05$).

also promotes epithelial-mesenchymal transition and metastasis in hepatocellular carcinoma via activation of JNK and NF-κB signaling [18]. Pei et al demonstrated that Rap2A enhances the migration and invasion of osteosarcoma cells and increases activities of matrix metalloproteinase MMP2 and MMP9 [7]. Moreover, our statistical analysis on clinical data suggested that Rap2A was in connection with HCC patients' tumor metastasis and vascular invasion. Given the results of aforementioned studies, we speculated that Rap2A might indeed participate in the development and progression of hepatocellular carcinoma.

According to our present study, Rap2A promotes HCC metastasis and brings a poor outcome for patients. On the other hand, Wu et al reported that Rap2A inhibits glioma cell migration and invasion by down-regulating p-AKT and probably serves as a tumor suppressor in the pathogenesis of glioma [19]. This study maybe controversial for the gene functions of Rap2A in carcinogenesis. The different effect of Rap2A on cancer might be due to cell-specific activity and diverse signal transduction. Thus, further studies about Rap2A in HCC are needed. For instance, we can examine the effect of Rap2A on HCC cell migration and invasion in nude mice using a heterotopic xenograft model.

In conclusion, our study demonstrated that high expression of Rap2A was correlated with poor prognosis of patients with HCC and Rap2A may be a predictive biomarker for HCC.

Acknowledgements

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Disclosure of conflict of interest

None.

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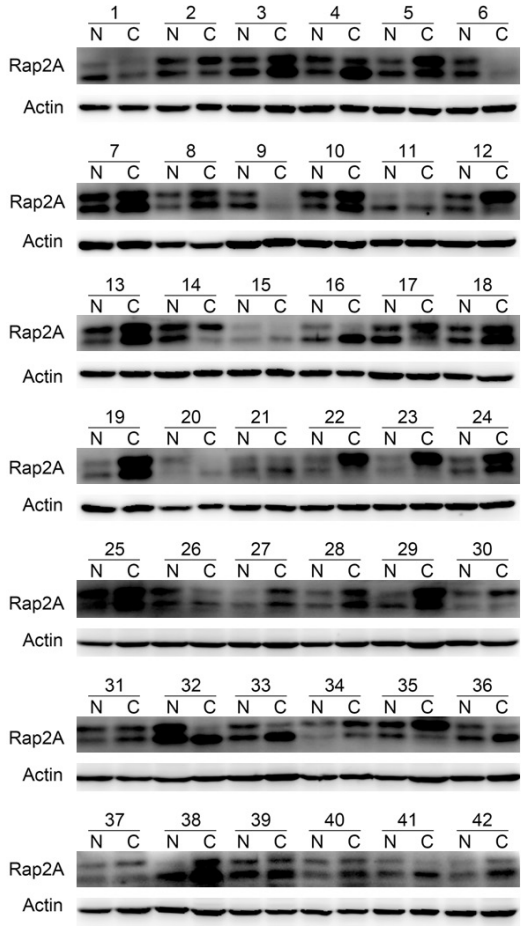

Figure S1. Expression of Rap2A protein in 42 paired HCC.

Table S1. Western blot band grayscale from 42 paired HCC tissues and adjacent normal liver tissues

Case	Actin normal	Rap2A normal	(Rap2A/β-Actin) normal	Actin cancer	Rap2A cancer	(Rap2A/β-Actin) cancer
1	176.129	50.346	0.285847305	166.08	22.969	0.138300819
2	160.06	74.038	0.462564038	169.373	62.017	0.366156353
3	233.5	72.94	0.312376874	224.29	100.749	0.44919078
4	174.927	65.59	0.37495641	168.201	83.11	0.494111212
5	168.365	58.962	0.350203427	160.431	77.011	0.480025681
6	151.668	63.57	0.419139172	162.941	28.179	0.172939898
7	228.984	107.184	0.468085106	230.811	129.902	0.562806799
8	165.654	71.462	0.431393145	169.964	111.539	0.656250735
9	195.969	96.13	0.490536769	189.245	27.531	0.145478084
10	166.366	84.115	0.505602106	167.35	137.282	0.820328653
11	152.464	51.444	0.337417358	168	45.518	0.270940476
12	163.544	66.024	0.403707871	149.079	91.389	0.613023967
13	144.006	96.278	0.668569365	143.502	139.928	0.975094424
14	169.941	96.451	0.567555799	149.014	66.24	0.444521991
15	171.603	27.349	0.159373671	170.189	22.932	0.134744314
16	164.61	61.072	0.371010267	150.079	84.994	0.5663284
17	172.811	118.549	0.686003784	167.027	103.687	0.620779874
18	168.7	89.676	0.531570836	150.52	125.89	0.83636726
19	172.212	49.705	0.288626809	181.313	102.664	0.566225257
20	132.769	27.403	0.206396071	151.829	22.86	0.150564121
21	172.267	50.04	0.290479314	160.613	39.132	0.243641548
22	154.639	37.873	0.244912344	158.797	99.938	0.629344383
23	134.078	31.16	0.232402035	160.134	116.873	0.729845005
24	167.186	56.042	0.335207493	122.989	111.493	0.906528226
25	151.357	53.727	0.354968716	147.385	63.111	0.428205041
26	167.668	44.954	0.268113176	163.086	34.274	0.210159057
27	161.942	33.385	0.206154055	173.489	54.628	0.314878753
28	154.571	36.835	0.238304727	163.83	87.261	0.532631386
29	157.208	56.819	0.361425627	160.937	84.994	0.528119699
30	143.239	25.364	0.177074679	144.528	61.665	0.426664729
31	163.446	68.204	0.417287667	143.94	75.691	0.525851049
32	149.077	109.007	0.731212729	158.484	66.397	0.418950809
33	152.608	59.872	0.392325435	154.264	94.177	0.610492403
34	143.648	35.216	0.245154823	146.095	72.359	0.495287313
35	210.827	92.133	0.437007594	218.636	120.39	0.550641248
36	167.242	82.32	0.492220854	155.044	71.327	0.460043601
37	142.913	54.286	0.379853477	139.856	49.642	0.354950807
38	235.988	102.268	0.433361018	225.639	128.713	0.570437735
39	135.392	96.416	0.712124793	132.788	85.736	0.645660752
40	133.929	49.813	0.371935876	127.883	66.638	0.52108568
41	135.897	46.924	0.345290919	148.715	72.291	0.486104293
42	149.668	55.483	0.370707165	151.463	87.964	0.580762298