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Full Length Research Paper

Comparison of classifications of aptamers against *Vibrio alginolyticus* based on their primary and secondary structure

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As a novel method to detect the pathogen *Vibrio alginolyticus*, 45 aptamers were previously selected and tested. In order to better understand the properties of these aptamers, it was essential to classify these aptamers based on appropriate criteria. The primary structure of 45 aptamers against *V. alginolyticus* was analyzed by the software DNAMAN and these aptamers were classified into 11 groups as the usual method according to their homogenous tree. However, the secondary structure of aptamers within each group varied greatly, which implies that the classification based on their primary structure is drastically different from that based on their secondary structure. Since the spacial structure like secondary structure is the basis for aptamers to bind to their targets, analysis on and classification by their secondary structure should be necessary and would be a good way to elucidate the mechanism of the specific identification of their targets. Further analysis on the secondary structure demonstrated that the fixed sequences were involved in the formation of the secondary structure of aptamers and contributed to the complexity of the structure, which makes it possible to increase their identification abilities.

Key words: Aptamer, primary structure, secondary structure, *Vibrio alginolyticus*, classification.

INTRODUCTION

As the most abundant vibrio in the ocean, *Vibrio alginolyticus* is a pathogenic microorganism widely distributed in the seawater all over the world. Under certain conditions, *V. alrinolyuticus* can infect many saltwater cultured animals such as fish, shrimp and shellfish posing risk which leads to food poisoning. Therefore, rapid and sensitive detection of the microorganism is definitely necessary and fundamental to control the occurrence and prevalence of the disease caused by this pathogen. However, the detection of the pathogen is still suboptimal due to the requirement of long time and expensive equipment and inadequate sensitivity of the present detection approaches.

Systematic evolution of ligands by exponential enrichment (SELEX) is a technology established in 1990s for selection of high affinity receptors or aptamers from a random oligonucleotide library which can form a large number of three dimensional configurations therefore possess the capacity to bind to almost all kinds of target agents (Tuerk and Gold, 1990; Ellington and Szostak, 1990). When compared with antibodies, aptamers selected by SELEX have many advantages such as high affinity, selection *in vitro*, high stability and easy control. Hence, aptamers have been broadly used in the detection of varieties of targets such as viruses, proteins, tumor cells and microorganisms (Chen et al., 2007; Kyong et al., 2008; Kyung et al., 2007; Liu et al., 2010; Swee et al., 2009; Yeon et al., 2007). However, the application of SELEX in the fields of marine biology and aquaculture is still in the burgeoning stage.

Based on the previous selection of aptamers targeting the microorganism *V. alginolyticus* (Zheng et al., 2008, 2010), a series of aptamers were obtained and sequenced. Given a large number of aptamers with

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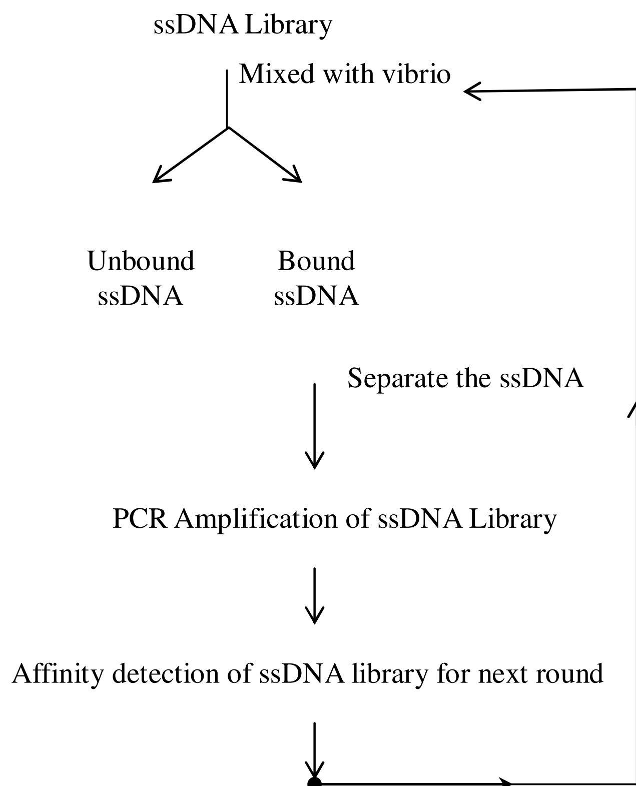

Figure 1. SELEX process.

various structures, it is absolutely essential to study their structure and classification in order to reveal the mechanism for specific identification of *V. alginolyticus.* Learning about the characteristics of structure and classification of aptamers will greatly facilitate our future finding of better candidates based on the structure. In the present paper, the primary and secondary structure of the aptamers against *V. alginolyticus* was analysed and classified in order to understand the characteristics of their structure, which could provide references for the research on selection of aptamers against pathogens.

MATERIALS AND METHODS

Forty five aptamers were obtained from the selection targeting *V. alginolyticus*. The selection was carried out according to the SELEX process shown in Figure 1 and described previously in detail (Zheng et al., 2010). The sequences of all the aptamers are made up of two parts: fixed primer regions at two ends and the middle regions (5′-TCA GTC GCT TCG CCG TCT CCT TC ----NNN….NNN---GCA CAA GAG GGA GAC CCC AGA GGG -3′, where the “N” in the middle region means any base of A, T, C and G). The fixed primer regions of the aptamers are the same in all molecules and used for binding with the primers at PCR process. The middle regions of the 45 aptamers are different and listed in Table 1. Because the length of the middle region in the initial SELEX library was 35 bases, most of aptamers have a middle region with 35-base length. Nonetheless, it is possible to have some aptamers with different lengths of their middle regions because the PCR process had nearly inevitably introduced some mutations. Therefore, the majority of aptamers have their middle regions with 35-base length, while the minority has other lengths.

The primary structure of the aptamers was analysed with the homologous tree being constructed by the software DNAMAN. Based on the homologous tree, the aptamers with close homology (more than 44%) were firstly clustered as a group. Thus, the aptamers were firstly divided into 10 groups. Then, the remaining aptamers 19 and 3 were clustered as the 11th group. In this way, the 45 aptamers could be classified into 11 groups according to their primary structure.

The secondary structure of the aptamers was simulated by the software RNAStructure based on their minimum free energies. According to the number of loops in the secondary structure of the middle sequences of aptamers, these aptamers could be classified into three types: single-loop, double-loop and three-loop structure. The last two types could then be classified into two subtypes: loops in series and in separation according to the relationships between loops. Loops with common side or connected only by dsDNA, are called loops in series, if not, these loops are in separation. Based on the number of loops in the secondary structure of the whole sequences of aptamers, these aptamers could also be classified into seven types: three-loop, five-loop, six-loop, seven-loop, eight-loop, nine-loop and ten-loop structure.

RESULTS

Analysis and classification of the primary structure

Since the sequences of fixed regions of the aptamers

Table 1. Sequences of the middle regions of the 45 aptamers.

S/N	Middle region	Length
1	GGCAGAGGAATACAGGGACGGGAGCACAAGAGGGA	35
2	GAGCGTCAGGGACAGAACGCTGGGGGACGGGGCGCACAAGAGGGAG	46
3	AGCCGAAGTGACGTAGCGATGTGGAGCTTGCAGAT	35
4	GGCGTAAGGTGAGGAGGAAGCGGCTGAGGGAGGCA	35
5	GCTACGAAGGAAGAAAGGCATTGAGCGGCTGAGGA	35
6	AGGGAACGGTAGGATAAGCGAATGACGGAGGGAGT	35
7	GGGGACTAGAAGGGACTGTCGACGATGGGATGGGA	35
8	AGAGTCGTGGAGAGGGTGAACGGAGGGGGGAAACA	35
9	AAAGTAGGTGAGGTCAGAGGGGACTGAACTGGGGA	35
10	AGGTGGCGAGTTGCGAAGGACTGTGTCGAGTGTTG	35
11	GGGGTGTAAGGCGACAGGGACTGTACGAGGGAGGA	35
12	GGCGTGACGACACGGAGTCAAAGTCAGCTAAGGTA	35
13	GGACGAGATGGGCGGGAAACGAGGCACAAGAGGGA	35
14	GGAAGCGGGAGGAGCGATGAGACCGAAAGGCGTG	34
15	CTTGTACCGGTAACACAAGGGGAGCACAAGAGGGA	35
16	CGAGAGGAATGGGCGAGAGACTATGTCGAGGATGG	35
17	GTTGTCGGTGAAGCAGGGAGGCAGCGCGGGAAGGG	35
18	GGGGAGGGCTCGAAGCCAGGCTGACGAAGAGGGGA	35
19	GTAACGAGGAACCGTAGGTTCAAAGTGGAGGACGA	35
20	GGGGGAAGACTGCGGCGGGCGGCGTGAGGGGAACG	35
21	CGGGGGACGATGGGCGAGGCAAGACGGTCCCGCTG	35
22	GTAGAAGGGCGCGAGTGGCTGAACGGAGGGAGGGA	35
23	AGGTTGCGTGCGGAGCACGTGAGGGAGCCGAGT	33
24	GGACCCTGTGGCGAGAGACGTAGGGGTAGGGAGGA	35
25	CCCGGGAGGGCATGGGCAATGACGGCTGGGAGAG	34
26	GGAAGCGGGTGGGAGAGGAGCGGAAGGTGAGTGAG	35
27	GGAGTGGGAGCAGCGATGCGGCGTATGACTGATTG	35
28	GTAGGGGCTAGCGGCTTAAGTGGGCACAAGAGGGA	35
29	GGCCGTGGGAATCGGATGATGAGCTAACGGAGGGA	35
30	AGGATTGACGGAGTGCGAGTGGTTGAGCGGATGTA	35
31	CAGGGGCGGGAAGAGGCTGACATGGATGGAGGTA	34
32	GCGGTACCACGGGTTGAGCGGTGGAAGTAGAGCGG	35
33	GGGCACTCGCGCGAGGGGCGGGGACTGGGACTGAGGCACAAGAGGGA	47
34	ATCGAAGATGAGCAACGGCGACGACGGTCACGAGTG	36
35	TGGAGGCAGGGCGCGACGTGAGGGGCCCGAGTACGA	36
36	ATGGGGTTAGGCGGGCGGGTGGTCGAGTCGGAGTA	35
37	AGCGGGATGAGGGAGTAGGAGGGCCACAGTGGACT	35
38	GGGGACATAGGGCATGACTGAGACTGGACGAGGA	34
39	GCAGGTGGGGGACGGAGCGACGGACGCGGGGAGTA	35
40	GGGGCACGAGGAAAGGGGAGGAGCAGAATGAG	32
41	GTGGGGCTGCGTGAGCGGGTGAGGCGACAGATTGG	35
42	GCGATACTGGCGTTAGCGGCTGGTCAACCGAAGTG	35
43	CAGGGGAGGTGTACGGGACTACGGTCGGGGGTGAG	35
44	GCAGGTGAAGGAACGGTAGCGGCTGAGGAAGGGGA	35
45	CGAGGTGGAGAGTTAGCGAGTGAGTCGACAGAGTG	35

were identical and the sequences of the middle regions varied among distinct molecules, the primary structure of the aptamers depends on the middle sequences. The middle sequences of the 45 aptamers were analysed and the homologous tree was constructed both by the software DNAMAN (Figure 2). Based on the homologous tree, the 45 aptamers were divided into 11 groups (Table 2). Groups 1, 4, 5, 9 and 11 have conserved sequences,

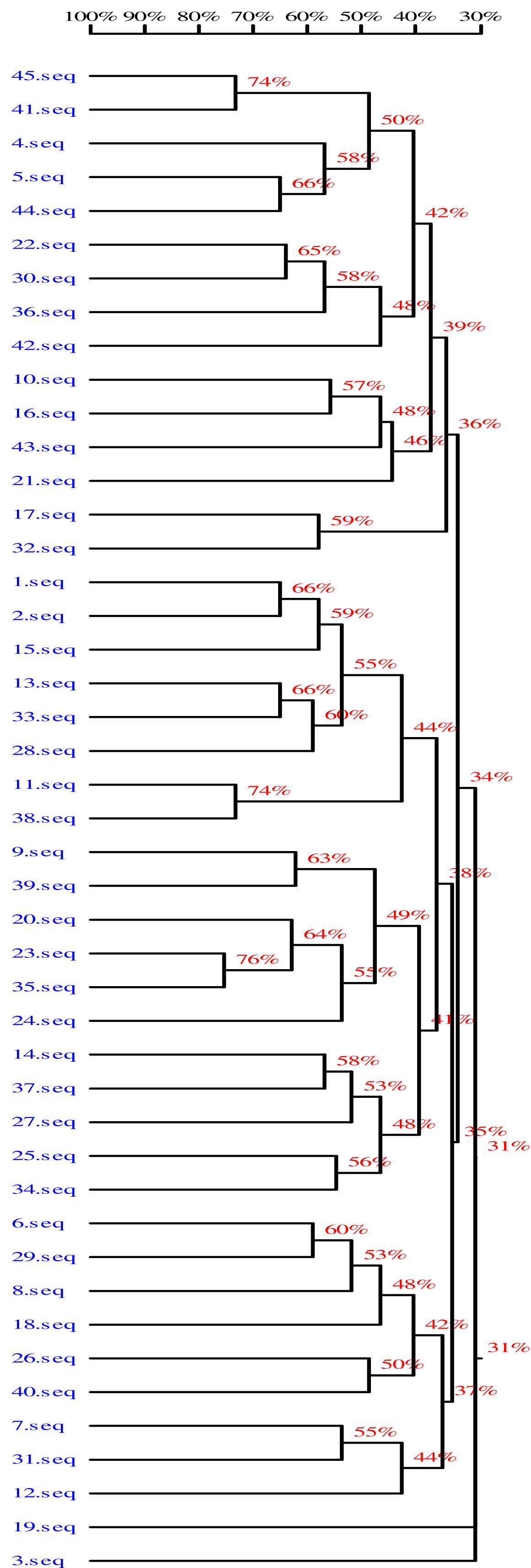

Figure 2. Homologous tree of the aptamers.

Table 2. Classification for aptamers based on their primary structure and the homogenous tree ("N" in the conserved sequences means any base of A, T, C and G).

Number of group	Aptamer numbers	Conserved sequences
1	4, 5, 41, 44, 45	AGCGNNTGAG
2	22, 30, 36, 42	None
3	10, 16, 21, 43	None
4	17, 32	GGT, AGC, GGA, GCGG
5	1, 2, 11, 13, 15, 28, 33, 38	ANGAGG
6	9, 20, 23, 24, 35, 39	None
7	14, 25, 27, 34, 37	None
8	6, 8, 18, 29	None
9	26, 40	AGNNGANTGAG
10	7, 12, 31	None
11	3, 19	CGA, CGTAG, GA

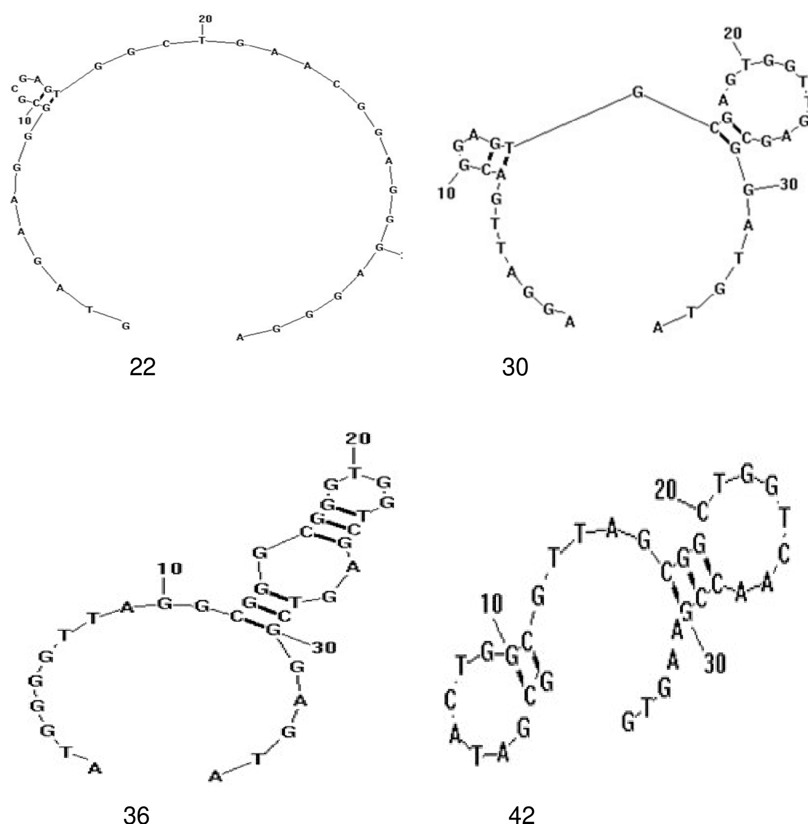

Figure 3. Secondary structure of the middle sequences of 2nd group of aptamers.

but other groups do not share obvious conserved sequences.

Analysis of the secondary structure of the middle sequences

Since the secondary structure of aptamers strongly contributes to the spacial structure of binding between aptamers and targets, and the differences in aptamers actually stem from the variations in the middle sequences, it is essential to study the secondary structure of the middle sequences of aptamers. Taking the 2nd group of aptamers as an example (Figure 3), the secondary structure of all aptamers within the group exhibits the complicated stem-loop structure. According to the number

Table 3. Distribution of secondary structure of the middle sequences of the 11 groups of aptamers.

Group number	Characteristics of the secondary structures					
	Single loop	Double loops		Three loops		N/A
		In series	In separation	In series	In separation	
1	5, 41, 45	4, 44	-	-	-	-
2	22	36	30, 42	-	-	-
3	16	-	10, 43	21	-	-
4	-	17	32	-	-	-
5	28, 38, 2, 15	11, 13	-	-	33	1
6	39, 35	-	20, 23	9, 24	-	-
7	27	-	37	14, 25	34	-
8	8, 18	6, 29	-	-	-	
9	40	-	-	-	-	26
10	-	12	31	-	7	-
11	19	3	-	-	-	-

N/A: No secondary structure could be simulated for the aptamers by the software RNAStructure.

of loops in the secondary structure of aptamers, these aptamers could be further classified. Aptamer 36 has two loops that are connected by double strand DNA, so the two loops are in series. Both aptamers 30 and 42 have two loops that are connected by ssDNA partly, so the two loops are in separation. Thus, the distribution of the secondary structure of the middle sequences of the 11 groups of aptamers could be obtained (Table 3). As shown in Table 3, the secondary structure of the aptamers in the same group is quite different. For example, the secondary structure of middle sequences of the 1st, 2nd, 8th and 11th groups of aptamers has two types (single-loop and double-loop), while the secondary structure of middle sequences of the 3rd, 6th and 7th group of aptamers manifests all three types. Besides the three types described earlier, a type without secondary structure was also found in the 5th and 9th groups of aptamers because no secondary structure of the middle sequence of the aptamer 1 and 26 could be simulated by the software. In summary, the secondary structure of the middle sequences within the same group of aptamers varied greatly, which suggests that the classification based on the primary structure of aptamers is very different from that based on secondary structure of their middle sequences.

Analysis of the secondary structure of the whole sequences

Besides the middle sequences, the whole sequences of aptamers also contain the two terminal fixed sequences, so it is not enough to just analyze the secondary structure of the middle sequences. The secondary structure of the whole sequences was studied so as to fully understand the characteristics of the secondary structure of aptamers. The secondary structure of the whole sequences of the 2nd group of aptamers is shown in Figure 4 and other aptamers share similar complex structure. When compared with that of middle sequences (Figure 3), the secondary structure of the whole sequences (Figure 4) was much more complicated. There were 3 to 10 loops in the secondary structure of the whole sequences, but only 1 to 3 loops in that of the middle sequences. Both types of loops- loops in series and in separation were found in each aptamer in the secondary structure of the whole sequences, but we rarely saw both in one aptamer in that of the middle sequences. Particularly, the secondary structure of the middle sequences of aptamer 1 and 26 was not able to be simulated by the software, but the secondary structure of their whole sequences was simulated successfully which exhibited 8 loops in either of them. This strongly suggests that the terminal fixed sequences can play important roles in the formation of secondary structure of aptamers.

As shown in Table 4, all the aptamers could be classified based on their loop number in the secondary structure of the whole sequences and the aptamers had a normal distribution relative to the loop number. There were only 2 aptamers with less than 5 loops, 5 aptamers with more than 9 loops and 38 aptamers with 6 to 8 loops. However, the distribution of the aptamers categorized by their secondary structure was dramatically different from the classification based on their primary structure, which clearly indicates that the classification based on the secondary structure provides distinct information for future analysis of aptamers against *V. alginolyticus* in contrast to that based on the primary structure.

DISCUSSION

Aptamer pool selected from initial random oligonucleotide library includes billions of aptamers with varieties of

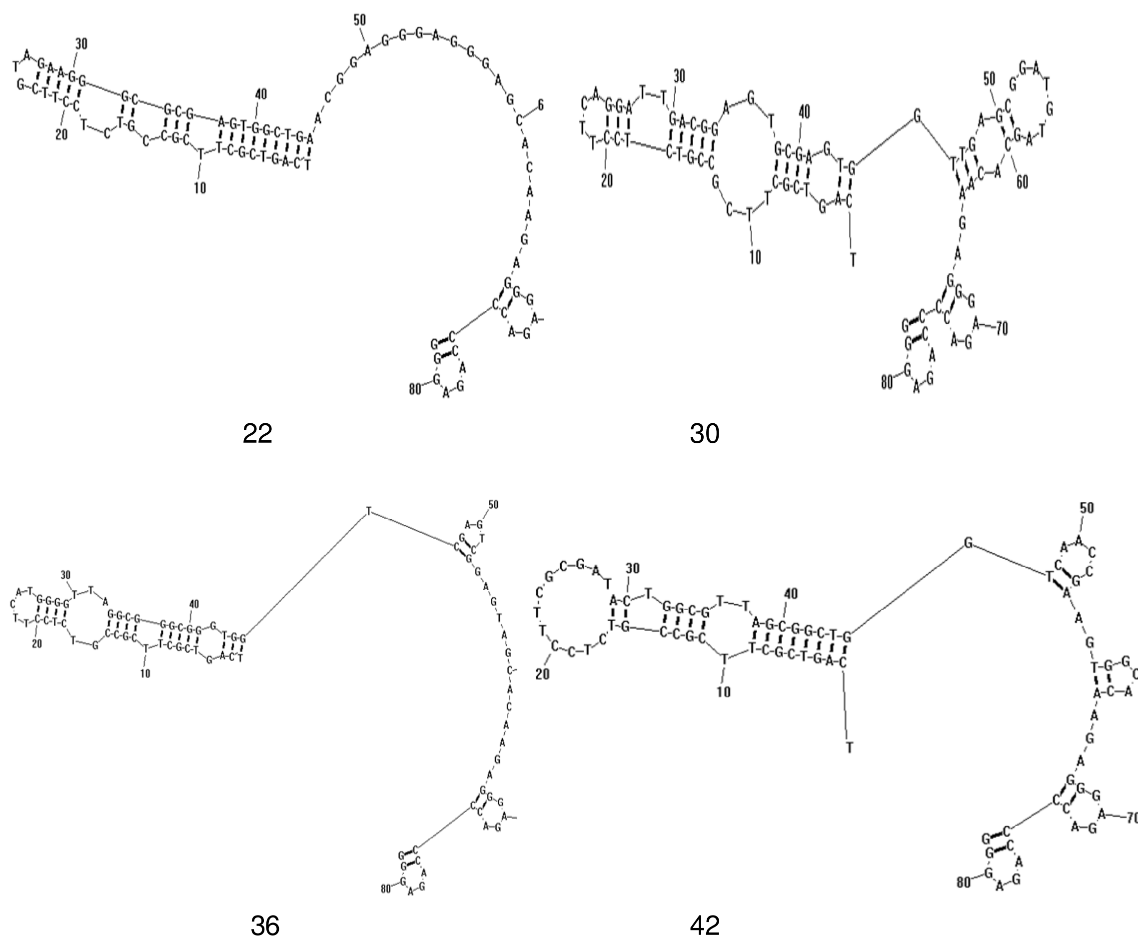

Figure 4. Secondary structure of the whole sequences of 2nd group of aptamers.

Table 4. Distribution of secondary structure of the whole sequences of the 11 groups of aptamers.

Group number	Number of loop						
	3	5	6	7	8	9	10
1	5		4, 44	41	45		
2			22	36, 42	30		
3				10, 43	16, 21		
4					17, 32		
5			2, 15, 28	13	1	33	11, 38
6			20, 23	24, 39	9, 35		
7			25, 37	27, 34		14	
8		8	6	18		29	
9			40		26		
10			12	7, 31			
11			3	19			

structure, therefore reasonable and efficient classification will facilitate our understanding of characteristics of aptamers' structure so as to reveal the mechanism for specific identification of target. The usual classification of aptamers is based on their primary structure, and those aptamers with closer homology in their primary structure are clustered as one group (Jacques et al., 2006; Zhan et al., 2005). Although, there is report revealing that the

similarity in the primary structure of aptamers followed that of their spacial structure like secondary structure to some extent (Zhan et al., 2003), some studies indicated that the similarity in the primary structure of aptamers did not necessarily dictate that in their secondary structure (Mohammad et al., 2004; Doerthe et al., 2005). In fact, some single stranded nucleic acids with highly conserved secondary structure in the evolution possessed obviously different primary structure (Michot et al., 1990; Bernard et al., 2000). The present research also proved that the classification based on the primary structure and the homologous tree was quite different from that based on their secondary structure. Theoretically, spacial structure like secondary structure is the basis for aptamers to bind to their targets. Therefore, classification only based on the primary structure of aptamers is not adequate. Analysis and classification based on the secondary structure of aptamers ought to be necessary and would be a good way to elucidate the mechanism of the specific identification of their targets.

Whether the terminal fixed sequences of aptamers should be included in their secondary structure simulation is still in dispute. Some studies showed that the fixed sequences had very limited effect on secondary structure and affinity of aptamers (Zeng et al., 2009; Tang et al., 2007). However, other studies proved that the fixed sequences had obvious effect on the secondary structure and affinity (Wang et al., 1999; Ei-ichiro et al., 2001; Mohammad et al., 2004; Doerthe et al., 2005; Sotiris et al., 2005). The present study strongly supported that the fixed sequences were involved in the formation of the secondary structure of aptamers and contributed to the complexity of the structure, which would be helpful to increase aptamers' capacity to identify their targets.

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