Abstract of Thesis

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Title	Studies on The Effect of Small Molecules on Trinucleotide Repeat Instability during DNA Replication Process (DNA複製時におけるトリプレットリピート不安定性に対する小分子の効果)

Trinucleotide repeat (TNR) is a repetition of triplet nucleotides such as (CAG)n, (CTG)n, (CGG)n, and (GAA)n. The expansion of TNR exceeding a certain threshold causes several incurable neuromuscular and neurodegenerative diseases, including Huntington disease, myotonic dystrophy, fragile X syndrome and many more. TNRs are transmittable to the offspring and tend to expand dynamically through generations, increasing the severity and the age of onset of the diseases. Contraction of the repeat length would alleviate the diseases progression.

We have sought small molecules targeting the TNR that can contract the repeat length, which could provide a potential therapeutic approach to cure such diseases. Nakatani group has successfully developed several small molecules that can selectively bind to the CXG repeats and induce the formation of hairpin structure. One of those ligands which is known as naphthyridine-azaquinolone (NA), could bind with high affinity to the CAG-CAG triad. This binding involves hydrogen bonding recognition of guanines and adenines by 2-amino-1,8-naphthyridine and 8-azaquinolone, respectively. We chose NA as a CAG repeat binding ligand and investigate how NA binding affects repeat instability during replication process. In this study, the ability of NA to induce repeat instability was evaluated.

A general introduction to the whole framework of this study is described in chapter 1. In chapter 2, the effect of NA on CAG_{20}/CTG_{20} repeat in *E. coli* cells was investigated. Gel electrophoresis and next generation sequencing (NGS) results showed that there are no significant changes in repeat length by the presence of NA as compared to that in the absence of NA, where 20 repeats remain unchanged. We hypothesized that this was due to the *E. coli* drug resistance against NA by the expression of efflux pumps. In chapter 3, we further replicated CAG_{20}/CTG_{20} repeat in an efflux pump-deficient (*ΔacrBΔtolC*) *E. coli*, where NA cannot be expelled from the cells. However, no significant change was observed in the repeat length even in the presence of NA. This might be due to the stability of CAG_{20}/CTG_{20} duplex in *E. coli*, as short repeats (30 repeats and below) were reported to be stable in *E. coli*. Therefore, any changes occurred might be too small to be identified.

In chapter 4, we tested the effect of NA on CAG_{20}/CTG_{20} repeat in an artificial replication process, using polymerase chain reaction (PCR). CAG_{20}/CTG_{20} was amplified by PCR in the presence of NA. The PCR amplified products were analyzed by gel electrophoresis and NGS. In the absence of NA, no significant repeat contraction can be observed. In contrast, repeat contraction occurred in a NA-concentration dependent manner. Interestingly, no contraction was observed when using neither negative control ligand that does not bind to the repeat nor changing the repeat sequence. In short, we have successfully demonstrated that NA induces CAG_{20} repeat contraction during PCR amplification. Changing NA with other repeat binding ligands, resulted in different contraction profiles. The contraction efficiencies and length distributions differ depending on the ligands. These findings offer a ligand screening method where it possibly proves that the ligands are not only binding to the repeat but also inducing repeat contraction.

In chapter 5, we examined how repeat length affects the contraction product profiles. In the presence of NA, contracted products were observed for templates having 16 to 21 CAG/CTG repeats. Besides, we have found that no contraction occurred when a template containing 10 repeats was used.

The contracted products differ in their distribution, depending on their respective templates repeat length. Broader distributions were observed by increasing the repeat length. For all the templates, the contracted products were observed to contain at least one repeat. Higher frequencies of contracted products having one repeat were observed for odd repeat templates (CAG_{17}, CAG_{19}, CAG_{21}) as compared to even repeat templates (CAG_{16}, CAG_{18} and CAG_{20}). These results explained the underlying mechanism of repeat contraction in the presence of binding ligands.

In chapter 6, the efficiency of Ion PGM to sequence CAG/CTG repeat in different length (10, 20 and 30 repeats) was analyzed. The efficiency and fidelity decrease by increasing the repeat length and by changing CAG to CTG repeat. Therefore, careful consideration should be given in using CG-rich template for Ion Torrent PGM sequencing and thorough analysis is required during data handling

We have successfully demonstrated that CAG/CTG repeat-targeting-ligands could induce repeat contraction during PCR amplification, where the contracted product distributions vary depending on the ligands used. Any ligand that can affects the instability and induces a contraction of the repeat length, has a therapeutic potential for the incurable repeat diseases. The PCR-based method demonstrated here would be useful for screening potential compounds for the development of functional ligands and drugs. This work provides an insight in understanding the mechanism of repeat contraction in the presence of repeat-targeting binding ligands.

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論文審査の結果の要旨及び担当者

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論文審査の結果の要旨

トリプレットリピート病は、トリヌクレオチド反復配列（TNR）の伸長によって引き起こされる一連の遺伝性障害である。例えば、CAGリピートは、その伸長によってハンチントン病等の神経変性疾患を誘発する。申請者は、TNRに特異的に結合する種々の合成リガンドが、TNRのヘアピン構造を安定化することにより、DNAの複製中にDNA鎖が短縮する可能性に着目し、DNA複製において、リガンド結合によりTNRの短縮、もしくは伸長の度合いの定量的評価に関する研究に取り組み、以下の成果を上げている。

1）大腸菌内におけるCAGリピート結合分子ナフチリジン−アザキノロン（NA）のプラスミド上に導入されたCAG20/CTG20リピートの短縮、伸長におよぼす影響の定性評価

大腸菌DH-5αに、CAG20/CTG20を持つプラスミドを導入し、一定時間の培養後のリピート長を調べたところ、NAの存在に関わらずリピート長の変化を観測しなかった。分子NAの菌内からの排出により効果が認められなかったという仮説を立て、排出トランスポーターacrB, tolCの欠損大腸菌株を用いてこの仮説を検証したが、分子NAの効果が認められないことを確認した。短縮、増幅効率の低さを補う高感度検出法が、課題となることを明確にした。

2）PCR増幅を用いるTNR配列の短縮・伸長の高感度検出系の開発

分子NAのTNR配列の短縮・伸長におよぼす効果を高感度に検証するために、僅かな短縮、伸長を増幅して検出できるPCRによる増幅過程をとりいれた高感度検出系を開発した。リピート結合分子NA共存下にCAG20/CTG20を含む領域をPCR増幅すると、2〜3リピートと極端にリピート長が短縮されたPCR産物が生成することを見出した。PCR産物のゲル電気泳動、配列解析、CAGリピート結合分子NAの濃度変化及び誘導体構造変化に対応したPCR産物生成との相関結果から、結合分子がCAGリピートに結合する事によりリピートの短縮したDNAが生成し、PCR過程で増幅されていることを明らかにした。

3）NGSを用いたリピート結合分子の定性、定量スクリーニング法の開発

複製過程における分子NAのリピート短縮効果をより定量的に評価するために、PCR産物の各リピート配列の生成頻度を、次世代シーケンサ（NGS）を用いて定量的に解析した。また、NAの誘導体、CTGリピートに結合する候補分子等について、その短縮効果を解析したところ、分子NAと同じくCAGリピートに強く結合する分子は、リピート長を極端に短縮すること、CTGリピート結合分子はリピート長の短縮は観測されるものの、リピート長の分布が広いことなど、リピート配列への結合様式との相関を示唆するデータを得た。これらの結果から、リピート配列が形成するヘアピン型二次構造が結合分子により安定化され、部分伸長した配列がヘアピンステムを越えてハイブリダイズすることが短縮する原因であると結論づけた。

上記の成果は、TNR結合分子のリピートへの結合強度、結合様式により微妙に変化するリピートの二次構造を、PCRによる短縮リピートDNAの増幅産物として読み取ることが出来ることを明らかにするとともに、疾病関連TNRに結合する低分子の網羅的探索の可能性示しており、高く評価できる。

よって本論文は博士（理学）の学位論文として十分価値あるものと認める。